昭和 32 年 7 月 9 日 第三種郵便物認可
第46巻 第10号 昭和34年10月25日発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 10, October 25, 1959

Vol. 46, No. 10 **October, 1959**

THE JOURNAL

OF

BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle, Rutlant, Vt., U.S.A.
Maruzen Co., Ltd., Tokyo, Japan
Santo Vanasio, Köln, Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥ 3,600 (U. S. $ 10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥ 3,600 (U. S. $ 10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U. S. A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co., Ltd.*, Chuo-ku, Tokyo, Japan.

The Journal of Biochemistry, Vol. 46, No. 10, 1959

TRYPSINOGEN-KINASE IN ASPERGILLUS ORYZAE

III. PURIFICATION OF TRYPSINOGEN-KINASE AND ITS RELATION TO ACID-PROTEASE

By KAZUO NAKANISHI

(From Takamine Laboratory, Sankyo Co., Ltd., Tokyo)

(Received for publication, December 1, 1958)

Kunitz (*1*) reported that a species of *Penicillium* producted an enzyme which activated trypsinogen (Tn) to trypsin (T) between pH 2.5 and 4.0. The author (*2*) also observed the presence of an enzyme with similar activity in the extracts of wheat bran-cultures of *Asp. oryzae* and its preparation, Takadiastase (Sankyo). He further reported a liberation of aspartic acid as the mechanism of the Tn-activation by this enzyme, and presumed this enzyme to be an acid protease. Desnuelle (*3*), investigating the mechanism of Tn-activation, confirmed the N-terminal amino acid of Tn and T to be valine and isoleucine respectively and suggested that a peptide containing a N-terminal valine was released from the N-terminal of Tn. Neurath (*4*) has, in fact, obtained a peptide containing a N-terminal valine (Val : Lys : Asp = 1 : 1 : 4) in activation of Tn by T. Desnuelle (*5*) investigated the amino acid construction of the N-terminal group of Tn by the DNP-method. The results obtained by these investigators have demonstrated that the peptide released in the Tn-activation has the structure Val-$(Asp)_4$-Lys. As regards the case of activation by enterokinase, Yamashina (*6*) found that the N-terminal of T produced by enterokinase was isoleucine, and accordingly presumed that a Val-peptide was released from the N-terminal of Tn as in the case of Tn-activation by T.

According to Miura (*7*) and Crewther (*8*), at least three different sorts of protease acting in alkaline, neutral or acidic media res- pectively are presumably present in Takadiastase.

Acid-protease is considered by Isojima (*9*) to be carboxypeptidase, an enzyme which despecializes horse serum, and he has reported a method to purify this enzyme. Yoshida (*10*) has obtained acid-protease in crystalline form from *Asp. niger* by a method using ion exchange resins.

To confirm the mechanism of the activation previously reported by the author and to compare his results with those of Neurath and Yamashina, purification of this enzyme was undertaken. Yoshida's method was found to be quite an easy method of purification. The purity of the refined substance increased almost 12-fold as compared with crude Takadiastase. The author was able to obtain a preparation of homogenous nature as demonstrated by paper electrophoresis. As the ratio between the activities of acid-protease

and trypsinogen-kinase was almost constant, during all the stages of the purification process, it was concluded that the acid-protease and trypsinogen-kinase were identical substances.

EXPERIMENTALS

1. Determination of Acid-Protease Activity—

Substrate (Hammarsten Casein, Merck, dissolved in HCl, adjusted to pH 3.0, 2 per cent solution) ..	1.5 ml.
Buffer solution (Mac Illvaine, pH 3.0)	0.5 ml.
Enzyme solution (diluted with $M/300$ KH_2PO_4)	1.0 ml.

After incubating above solution at 35° for 10 minutes, the solution was mixed with 3.0 ml. of 10 per cent trichloroacetic acid and incubated again for 20 minutes at 35° and then filtered. To 1.0 ml. of the filtrate 5 ml. of 0.4 M Na_2CO_3 and 1.0 ml. of Folin reagent (diluted 5 times) were added and after standing at 35° for 20 minutes, the optical density was measured at 660 mμ. This value, after subtracting the control value (blank), was taken as the activity. This optical density exactly present the amount of acid protease when it is below 0.1.

2. Determination of Trypsinogen-Kinase Activity—In 10 ml. of 80 per cent glycerol 0.5 g. of acetone powder of pig pancreas was dissolved and left standing at room temperature for 2 hours. 10 ml. of water was then added to this glycerol solution and filtered. 4.0 ml. of MacIllvaine buffer solution (pH 3.5) was added to 1.0 ml. of the filtrate and the resulting solution was used as the trypsinogen.

Trypsinogen ..... 0.5 ml. Kinase ($M/300$ KH_2PO_4 dilution) 0.5 ml.	activated at 35° for 20 minutes	at 35° for 10 minutes
Casein 2 per cent (Casein 2 g./0.1 M Na_2HPO_4) 0.1 M K_3PO_4	equal volume mixture ..... 2 ml.	at 35° for 10 minutes

After adding 3 ml. of 10 per cent trichloroacetic acid, the procedure was the same as that in the case of acid-protease. For control experiments, kinase was added after the addition of trichloroacetic acid. The difference, *i.e.* the amount of trypsin activated was taken as the activity of the kinase. This optical density exactly present the amount of the kinase when it is below 0.2.

3. Protein Content—Protein content was measured according to Lowry's method (*11*) and presented by optical density.

4. Color Density—The color density was presented by optical density at 470 mμ.

5. Ion Exchange Resin—Duolite CS 101 (Na-form) was dried, pulverized, and passed through a 70 mesh screen. The particles were suspended in water and left standing for 30 minutes. After that the precipitated particles were taken. The same procedure was repeated several times. After washing with water, the resin was buffered to the required pH.

As acid-protease and trypsinogen-kinase were considered to be identical substances in preliminary experiments and the former is more easily measurable, purification of the acid protease was carried out first and then acid-protease and trypsinogen-kinase were measured during each stage of the purification process and their ratio was calculated.

Experiment I. Adsorption of Acid-Protease at Various pH

A 20 per cent solution of Takadiastase was dialysed overnight by a col-

lodium membrane against $M/20$ acetic acid buffer solution of pH 4.7 in the refrigerator and the inside solution was used as the adsorption stock solution. Solution of different pH were prepared by adding HCl to this stock solution. To each 5.0 ml. of these solutions, 2 g. of ion exchange resin buffered respectively to the same pH were added, and the mixture was shaken for 30 minutes and centrifuged. Activity of the supernatant was then compared with that of the stock solution. The results are shown in Table I.

TABLE I

Adsorption of Acid Protease by CS 101 at Various pH

	Before adsorption			After adsorption		
pH	3.3	3.7	4.2	3.3	3.7	4.2
Activity	7.60	7.60	7.60	1.26	1.44	2.40
Adsorption %				83%	81%	68%

To 5 ml. of the stock solution adjusted to various pH, 2 g. of resin buffered to the same pH was added and the solution was shaken for 30 min. The activity of supernatant was measured.

Almost no change in the adsorption power was observed between pH of 3.3 and 3.7. At pH 4.2, however, the adsorption power seemed to be somewhat lower. Since the enzyme itself became unstable at pH 3.0, all succeeding experiments were carried out at pH 3.5.

Experiment II. Adsorption on Ion Exchange Resin-Column

Experiment I showed that the optimum pH was 3.5. The dialysis was omitted in this experiment as it was found that this enzyme was inactivated partially during this procedure. Instead of dialysis to lower the ionic strength, the concentration of the Takadiastase extract solution was lowered to 2 per cent, and the pH was adjusted to 3.5 with HCl. The solution was then filtered through cellite to remove the slight cloudiness, and the filtrate was used as the adsorption stock solution. The resin column was prepared by packing 30 ml. of the resin into a glass tube, 22 cm. in height with an inside diameter of 1.2 cm. The rate of pouring was 80 ml. per hour. The effluent was divided into 7.0 ml. and the protease activity of the 10th, 20th, 30th and 40th tube was measured. The results are given in Fig. 1.

If 144 ml. is to be taken as the end point of adsorption, the overall adsorption will be reached to 90 per cent.

Experiment III. Elution from the Adsorption Column

Elution was carried out with 0.5 M CH_3COONa after gently washing the resin column on which protease was adsorbed in *Experiment II.* The rate of elution was 50 ml. per hour. The effluent was divided into 4.0 ml.

and the protein content, protease content, pH and color density of each portion were measured. The results are shown in Fig. 2.

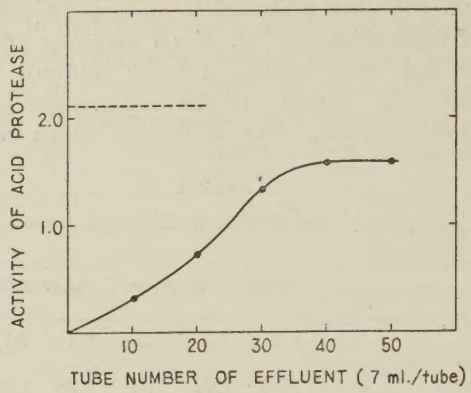


FIG. 1. Adsorption on ion exchange resin column.

The stock solution was 2 per cent Takadiastase solution with pH adjusted to 3.5. The solution was passed through the resin, CS-101 (22×1.2 cm. 30 ml.) buffered to pH 3.5 at the rate of 80 ml./hour. The activity of the effluent was measured. (A) - - - - the stock solution, (B) —— the effluent.

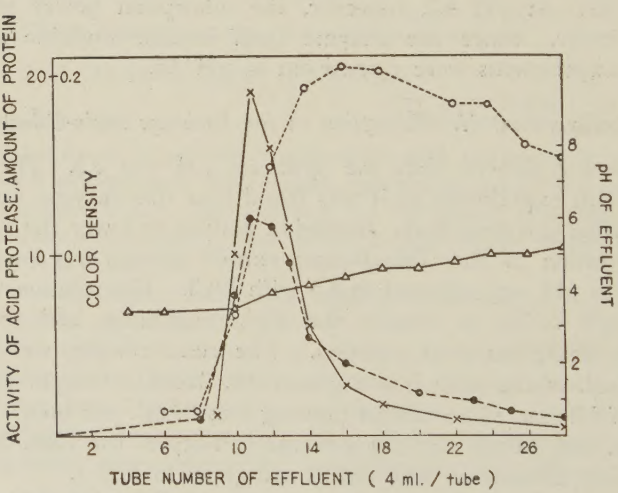


FIG. 2. Elution from the adsorption column.

Elution was carried out with 0.5 *M* CH_3COONa from the adsorption column mentioned in Fig. 1. The rate of elution was 50 ml./hour. The activity of acid protease (—×—), amount of protein (—●—), color density (—○—) and pH (—△—) of the effluent were measured.

From the point on which the pH of the effluent began to change, elution

of both protein and protease from the resin column commenced, first increased to their highest levels and then decreased. This tendency corresponded to the results obtained in Yoshida's study on the protease. The color of the effluent appeared slightly later after the elution of protein and protease began but the effluent remained colored even after protein and protease were almost eluted out. The ratio of protease to protein, *i.e.* the purity, was 1.5 which indicated approximately 5 to 6-fold increase as compared to 0.25 in the case of the adsorption stock solution.

Experiment IV. Purification and Ratio of Protease to Trypsinogen-Kinase

The above experiments showed that purification could be achieved to a considerable degree through the processes of adsorption and elution, so the purification was performed on a large scale as shown in Scheme 1. The acid protease, trypsinogen-kinase and protein contents of each fraction were measured and the results were shown in Table II. The highest degree of

SCHEME 1

Method of Purification

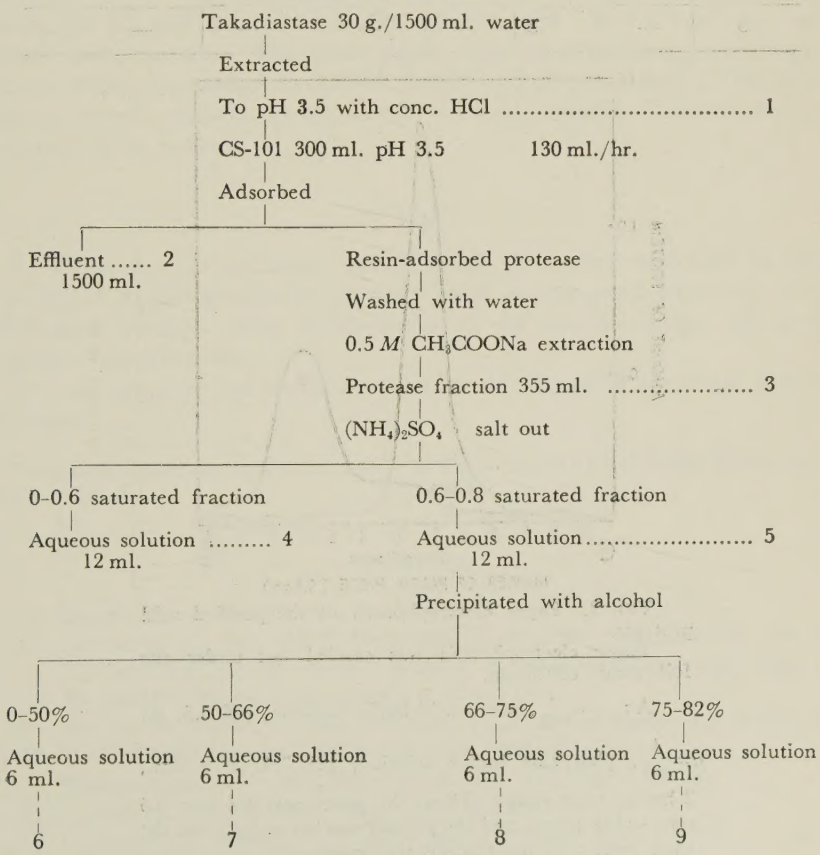


Table II

Purity, Yield and Ratio of Acid Protease to Trypsinogen-Kinase

Frac. No. in Scheme 1	Total protease (A)	Total protein (P)	Purity (A)/(P)	Yield of (A)	Total trypsinogen-kinase (K)	Purity (K)/(P)	Ratio (A)/(K)
1	3555	12690	0.28 (1.00)	100	6645	0.52 (1.00)	0.53
2	375	7005	0.053	11	—	—	—
3	1917	1455	1.31 (4.67)	54	3869	2.65 (5.09)	0.50
4	508	453	1.12	14	—	—	—
5	470	250	1.88 (6.71)	13	1068	4.27 (8.21)	0.44
6	147	93	1.58	4	—	—	—
7	202	77	2.62 (9.35)	6	474	6.15 (11.8)	0.43
8	—	6	—	—	—	—	—
9	—	3	—	—	—	—	—

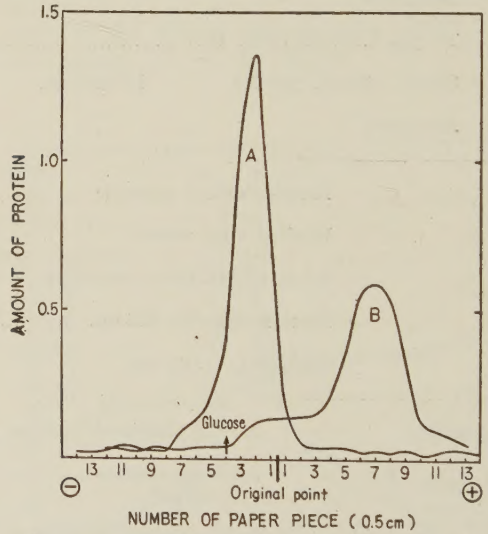


FIG. 3. Paper electrophoresis of the purified acid protease.

Paper electrophoresis was carried out under the following condition.

A: $\mu=\frac{1}{40}$, acetic acid buffer solution, pH 5.5, B: $\mu=\frac{1}{40}$, phosphate buffer solution, pH 7.4, 8 v./cm., 2 hrs. at room temp. Then, the paper was cut into 0.5 cm. -wide pieces and the protein was extracted from the each piece and the amount was measured.

purity was seen in the 50–66 per cent alcohol fraction. Compared to the Takadiastase extract solution, purification in respect to protease showed a 9-fold increase, and in respect to trypsinogen-kinase a 12-fold increase. The protease/kinase ratio decreased gradually by *ca.* 20 per cent from 0.53 to 0.43, but no marked change was observed. As mentioned previously three different sorts of protease exist in Takadiastase, and it is expected that, in the case of the crude sample, proteases other than acid-protease is partially included in the measurement of acid-protease. Therefore the result given here can reasonably be explained if it is presumed that among the 3 different sorts of protease only aci- protease exhibited the trypsinogen-kinase activity.

Experiment V. Paper Electrophoresis

In the same way as shown in *Experiment IV*, 40 mg. of dried purified acid protease was obtained from 30 g. of Takadiastase. This sample showed a protease/protein ratio of 2.4 and 4.3, units/mg. 7 mg. of this substance was dissolved in a few drops of buffer solution, and was subjected to paper electrophoresis under the following conditions: $\mu = 1/40$; acetic acid buffer solution, pH 5.5 and phosphate buffer solution, pH 7.4; 8 v./cm.; two hours at room temperature. The filter paper was cut into 0.5 cm. -wide pieces and each piece was soaked in $M/300$ KH_2PO_4 (3 ml.) overnight. The amount of protein in each extract was measured. As shown in Fig. 3 the results could be considered as uniform.

SUMMARY

1. Using the ion exchange resin method, the author succeeded in raising the purity of trypsinogen-kinase almost 12 fold as compared with the original Takadiastase powder. The homogeneity of the enzyme sample was proved by paper electrophoresis.

2. Acid-protease and trypsinogen-kinase of *Aspergillus oryzae* are identical substances.

The auther wishes to express his thanks to Prof. A k a b o r i of Osaka University for his kind guidance.

REFERENCES

(*1*) Kunitz, M., *J. Gen. Physiol.*, **21**, 601 (1951)
(*2*) Nakanishi, K., *Ann. Rep. Takamine Lab.*, **3**, 70 (1951); Nakanishi, K., *Ann. Rep. Takamine Lab.*, **4**, 89 (1952); Akabori, S., Nakanishi, K., and Fujii, K., *Symposium on Enzyme Chem. (Japan)*, **5**, 95 (1950)
(*3*) Rovery, M., Fabre, C., and Desnuelle, P., *Biochim. et Biophys. Acta*, **9**, 702 (1952)
(*4*) Davie, E. W., Neurath, H., *J. Biol. Chem.*, **212**, 515 (1955)
(*5*) Desnuelle, P., Fabre, C., *Biochim. et Biophys. Acta*, **18**, 49 (1955)
(*6*) Yamashina, I., *Acta Chem. Scand.*, **10**, 739 (1956)
(*7*) Motonaga, K., Miura, Y., *J. Agr. Chem. Soc. Japan*, **32**, 422 (1958)

(*8*) Crewther, W. G., Lennox, F., *Nature*, **165**, 680 (1950)
(*9*) Amano, T., Isojima, S., Fujio, H., *Med. J. Osaka Univ.*, **4**, 255 (1953); **5**, 333, 339 (1954)
(*10*) Yoshida, F., *Bull. Agr. Chem. Soc. (Japan)*, **20**, 252 (1956)
(*11*) Lowry, O. H., Rosenbrough, N. J., Forr, A. L., and Randall, R. T., *J. Biol. Chem.*, **193**, 265 (1951)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

STUDIES ON METABOLISM OF AMIDES IN MYCOBACTERIACEAE

III. AMIDASES AND TRANSFERASES IN EXTRACTS FROM MYCOBACTERIACEAE

By TOKUJI KIMURA

(*From the Laboratory of Biochemistry, College of Science, St. Paul's University, Tokyo*)

(Received for publication, December 8, 1958)

Up to the present, less attention has been paid to bacterial amidases than to animal amidases. In *Mycobacteriaceae*, Kirchheimer and Wittaker (*1*) reported the presence of asparaginase in various species, and Halpern and Grossowicz (*2*) studied amidases in extracts from *Mycobacterium phlei* and *Mycobacterium tuberculosis var. bovis*. Kimura (*3*) also reported on the highly purified nicotinamidase from *Mycobacterium avium*.

As for the transferase activity of *Mycobacteriaceae*, Grossowicz and Halpern (*4*) reported the presence of glutamotransferase in extracts from *M. phlei*. Recently Kimura (*5*) found a new transferase which catalysed the transfer of the nicotinyl group of nicotinamide to hydroxylamine.

This study deals with the distribution of amidase and transferase activities in three species of *Mycobacteriaceae*, and also with the enzymatic transfer of the acetyl group of acetamide to hydroxylamine in *M. smegmatis*.

METHODS AND MATERIALS

Quantitative Analysis—The rate of deamination of amides was determined by measuring the amount of ammonia formed, which was determined by Conway's micro-diffusion method. Hydroxamate was electrophotometrically measured by the color of the ferric complex after the Lipmann-Tuttle method (*6*). The protein concentration was measured by Lowry's method (*7*).

Assay of Enzyme Activity—For measurement of amidase activity, the standard assay mixture contained in 2.0 ml.: 50 μM of Tris buffer (pH 8.0), amides and the enzyme. For measurement of transferase activity, the standard assay mixture contained in 4.0 ml.: 200 μM of phosphate buffer, amides, 200 μM of hydroxylamine, and the enzyme.

The reaction was started by the addition of the enzyme, and the incubation was carried out for 60 minutes at 38°. The blank control, which contained all components except the substrate, produced negligibly small amount of ammonia or hydroxamate.

Materials Used—The nicotinamide (m.p. 129–130°), benzamide (m.p. 127–129°), L-asparagine, L-glutamine, and acetamide were obtained from commercial sources. The acetamide (m.p. 76.5–81.5°) was once recrystallized.

Preparation of the Enzyme—*Mycobacterium avium* (strain Takeo), *Mycobacterium phlei* and

Mycobacterium smegmatis were grown in glycerol-bouillon medium for 5 days. The harvested cells were washed and drained as much as possible and then poured into 10 volumes of acetone previously cooled to −40° by the addition of dry ice. After brief stirring, the cells were allowed to settle, the supernatant fluid was decanted, and the cells were desiccated *in vacuo* until the solvent was completely evaporated.

One gram portions of the acetone dried cells prepared from each of three species were then finely ground with a large amount of sea-sand, and extracted with 40 ml. of 0.1 *M* phosphate buffer (pH 7.0). After standing overnight in a refrigerator, the cells were centrifuged off at 10,000 r.p.m. for 20 minutes at 0°. The supernatant fluid was used as crude extract.

RESULTS

Distribution of Amidases in Mycobacteriaceae—The distribution of amidases in *Mycobacteriaceae* was tested with nicotinamide, benzamide, L-glutamine, L-asparagine, and acetamide as substrate. As shown in Table I, which summarizes the distribution of the enzymes, the extracts from *M. avium* hydrolysed nicotinamide, benzamide, and L-asparagine but not L-glutamine and acetamide. By the extracts from *M. phlei*, nicotinamide and L-asparagine were hydrolysed, while benzamide, L-glutamine, and acetamide were not hydrolysed. By the extracts from *M. smegmatis*, nicotinamide and benzamide were hydrolysed strongly, acetamide and L-asparagine moderately, while L-glutamine was not hydrolysed.

Grossowicz and Halpern (*2*) reported that the extracts from *M. phlei* were active towards L-asparagine, nicotinamide, and glycinamide, but inactive towards L-glutamine. These observations on *M. phlei* are consistent with our results.

TABLE I

Distribution of Amidases in Mycobacteriaceae

Substrate / Species	Specific Activity				
	Nicotinamide (200 μM)	Benzamide (25 μM)	L-Glutamine (50 μM)	L-Asparagine (50 μM)	Acetamide (200 μM)
M. avium	2.2	0.8	0	2.2	0
M. phlei	1.6	0	0	2.8	0
M. smegmatis	52.0	30.3	0	4.0	9.2

Extract; Tris buffer (pH 8.0), 50 μM; substrate as indicated. Total volume, 2.0 ml.; 38°, 60 minutes.
Specific activity = μM of NH_3 formed/hr./mg. protein.

Thus it became clear that the extracts from three species of *Mycobacteriaceae* were active both towards nicotinamide and towards L-asparagine and

were inactive towards L-glutamine. *M. smegmatis* had the strongest amidase activity among the species tested.

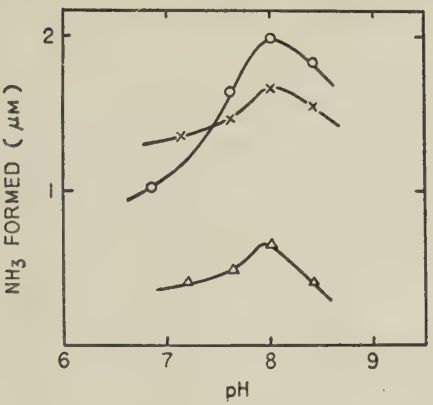


FIG. 1. Effect of pH on hydrolysis of amides in *Mycobacterium avium*. Dialysed extract 770 μg. of protein; Tris buffer (pH as indicated), 50 μM; total volume 2.0 ml.; 38°, 60 minutes.

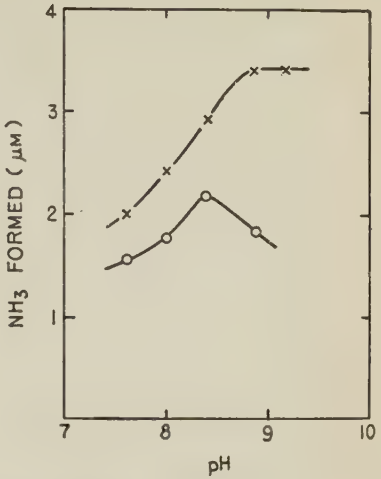


FIG. 2. Effect of pH on hydrolysis of amides in *Mycobacterium phlei*. Dialysed extract, 1120 μg. of protein; Tris buffer (pH as indicated), 50 μM; total volume, 2.0 ml.; 38°, 60 minutes.

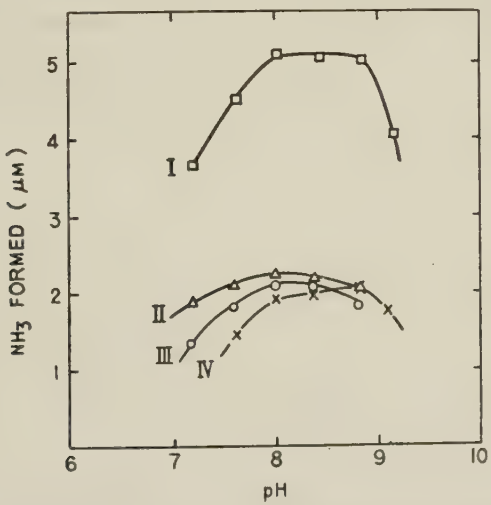


FIG. 3. Effect of pH on the hydrolysis of amides in *Mycobacterium smegmatis*.

Dialysed extract, 500 μg. of protein in Curve I, and IV; 65 μg. of protein in Curve II; 50 μg. of protein in Curve III. Tris buffer (pH as indicated), 50 μM; total volume, 2.0 ml.; 38°, 60 minutes.

TABLE II

Distribution of Transferase in Mycobacteriaceae

Source	Substrate	Nicotinamide (50 μM)	Benzamide (25 μM)	L-Asparagine (50 μM)	L-Glutamine (50 μM)	Acetamide (500 μM)
M. avium	Crude extract	1.71	0.81	0.05	0.00	0.83
	Protamine supernatant	1.96	0.70	0.00	0.00	0.80
M. phlei	Crude extract	0.00	0.00	0.00	0.12	—
M. smegmatis	Crude extract	0.36	0.49	0.04	—	4.45

In *M. avium*, Crude extract, (1250 μg. of protein)*
protamine supernatant (1130 μg. of protein)*.
In *M. phlei*, Crude extract, (2210 μg. of protein)*.
In *M. smegmatis*, Crude extract, (2490 μg. of protein)*.
Tris buffer (pH 8.0), 200 μM; substrate as indicated; NH_2OH, 200 μM; 38°, 60 minutes.

* The values indicate the amount of enzyme protein which was used for the assay.

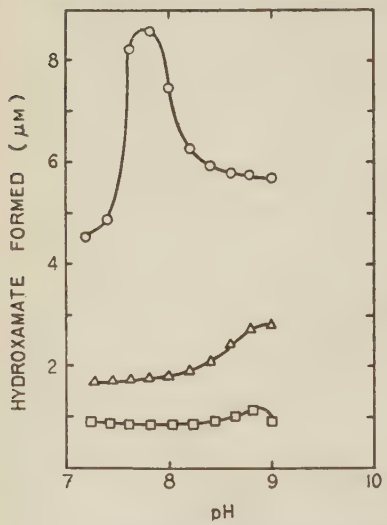


FIG. 4. Effect of pH on transferase activities in extract from *Mycobacterium avium*.
Extract, 1715 μg. of protein; Tris buffer, 200 μM; acetamide, 500 μM; benzamide, 25 μM; nicotinamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

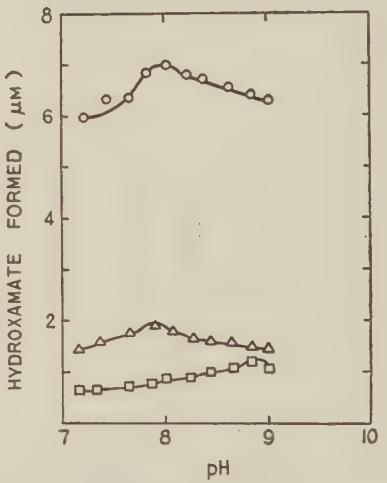


FIG. 5. Effect of pH on transferase activities in extracts from *Mycobacterium smegmatis*
Extract, 1150μg. of protein; Tris buffer, 200 μM; acetamide, 50 μM; benzamide, 25 μM; nicotinamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml; 38°, 60 minutes.

Fig. 1 shows the effect of pH on the hydrolysis of amides by the extract from *M. avium*. Optimum pH is about 8.0 for each of the substrates tested. Fig. 2 shows the case with the extracts from *M. phlei*. Optimum pH for nicotinamides is 8.3 and that for L-asparagine is near 9.0. Fig. 3 shows the results with the extracts from *M. smegmatis*. Optimum pH for nicotinamide (Curve III), benzamide (Curve II), L-asparagine (Curve IV), and acetamide (Curve I) is 8.0–8.8.

Distribution of Transferase in Mycobacteriaceae—The distribution of transferase in *Mycobacteriaceae* was tested with nicotinamide, benzamide L-glutamine, L-asparagine, and acetamide as substrates. As shown in Table II the extracts from *M. avium* were active towards nicotinamide, benzamide, and acetamide, while they were inactive towards L-asparagine and L-glutamine. The activities towards benzamide and acetamide were about the half as that towards nicotinamide. The extracts from *M. phlei* were generally weak in **transferase** activity, but slight activities towards L-glutamine was observed. The extracts from *M. smegmatis* were active towards nicotinamide, benzamide and acetamide.

Fig. 4 shows the effect of pH on transferase activities in extracts from *M. avium*. Optimum pH is 7.6–7.8 for nicotinamide, 8.8 for benzamide, and near 9.0 for acetamide. Fig. 5 shows the results with extracts from *M. smegmatis*. Optimum pH is 8.8 for acetamide, optimum pH is 7.8 for benzamide, and near 8.0 for nicotinamide when the protamine supernatant fraction is used.

Acetyl Transfer Reaction in M. smegmatis—With extracts from *M. smegmatis*, a transfer of the acetyl group of acetamide to hydroxylamine was observed. Fig. 6 indicates the rate of hydroxamate formation from acetamide as a

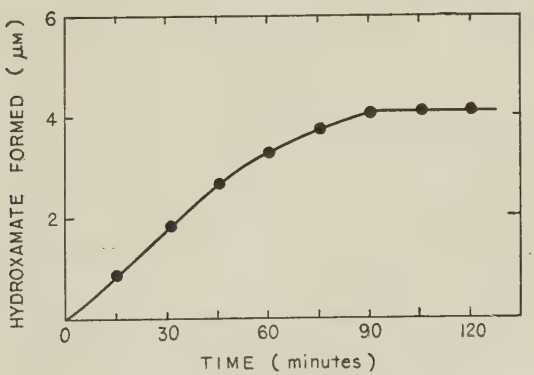


FIG. 6. Rate of hydroxamate formation from acetamide as a function of time in *Mycobacterium smegmatis*.
Extract, 1910 μg. of protein; Tris buffer (pH 8.0), 200 μM; acetamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

function of time. The equilibrium is attained in about 90 minutes. Fig. 7 shows the effect of substrate concentration on hydroxamate formation from acetamide. From the data obtained, Michaelis' constant can be tentatively

calculated as:

$$K_m = 4.4 \times 10^{-3} M.$$

This value is about 10 times larger than that for nicotinamide in *M. avium*. Table III indicates the heat inactivation of acetotransferase from *M. smegmatis*. This enzyme is stable to heat, compared with the nicotinotransferase from *M. avium*.

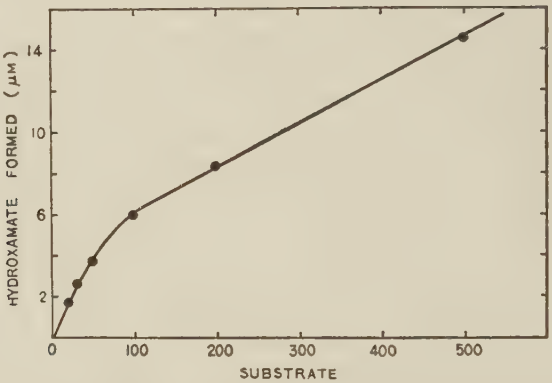


FIG. 7. Effect of substrate concentration on hydroxamate formation from acetamide in *Mycobacterium smegmatis*. Extract, 2840 μg. of protein; Tris buffer (pH 8.0), 200 μM; acetamide as indicated; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

TABLE III

Heat Inactivation of Acetotransferase from Mycobacterinm smegmatis

Treatment	Hydroxamate formed (μM)	Inactivation (%)
None	3.30	0
50°, 5 minutes	2.73	17
50°, 10 minutes	2.55	23
50°, 15 minutes	1.95	41
50°, 20 minutes	1.41	57
None	2.49	0
60°, 5 minutes	1.53	39
50°, 10 minutes	0.87	65
60°, 15 minutes	0.57	77
60°, 20 minutes	0.21	92

Extract, 2480 μg. of protein; Tris buffer (pH 8.0), 200 μM; acetamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml. 38°, 60 minutes.

DISCUSSION

Halpen and Grossowicz (2) studied amidases of extracts from *M. tuber-*

culosis var. bovis and *M. phlei*, and they were able to show that the extracts of either of these two species catalysed the hydrolysis of L-asparagine, leucinamide, and valinamide. They also found a slight activity towards L-glutamine, and no activity at all towards phenylaninamide. The extracts from *M. phlei* were also active towards glycinamide, formamide, and nicotinamide, but not towards benzamide. The extracts from *M. tuberculosis var. bovis* were inactive for all of these amides.

TABLE IV

Distribution of Amidase and Transferase in Mycobacteriaceae

Substrate \ Species / Activity		*M. avium*	*M. phlei*	*M. smegmatis*	*M. tuberculosis var. bovis* (2)
Nicotinamide	Amidase	+	+	+	
	Transferase	+	−	+	
Benzamide	Amidase	+	−	+	
	Transferase	+	−	+	
L-Glutamine	Amidase	−	−	−	−
	Transferase	−	+		
D-Asparagine	Amidase	+	+	+	+
	Transferase	−	−	−	
Acetamide	Amidase	−	−	+	
	Transferase	+	−	+	

+ ; presence of the enzyme activity,
− ; absence of the enzyme activity.

From the results obtained in this study also, it is concluded that *M. phlei* has weak amidase activities towards nicotinamide and L-asparagine, compared with *M. avium* and *M. smegmatis*. In addition to this, it is apparent that *M. avium* has at least two amidase activities, the one towards L-asparagine and the other towards nicotinamide and that *M. smegmatis* has strong amidase

activities towards nicotinamide and benzamide and fairly strong activities towards L-asparagine and acetamide.

Grossowicz and Halpern (*4*) showed that in *M. phlei* glutamotransferase exists but aspartotransferase does not and that glutamotransferase requires Mn^{++} and phosphate ions. It has been found in our laboratory also that in *M. phlei*, weak glutamotransferase exists but nicotintransferase does not. In *M. avium*, nicotinotransferase exists and its extracts are also active towards benzamide and acetamide, but inactive towards L-asparagine and L-glutamine. In *M. smegmatis*, a strong acetotransferase exists and its extracts are active towards nicotinamide and benzamide and slightly active towards L-asparagine.

Taking all these bindings into account, it can be concluded that the simultaneous existence of amidase and transferases is not always the ease in *Mycobacteriaceae*, as summarized in Table IV.

The enzymatic transfer of the acetyl group of acetamide to hydroxylamine has not been reported before. However, the physiological significance of this enzyme is not clear from the present data. The purification of this enzyme is now in progress, and the details of the properties of the enzyme will be published in another paper.

SUMMARY

1. The occurrence of amidases and transferases has been demonstrated in extracts from *M cobacterium avium* (strain Takeo), *Mycobacterium phlei*, and *Mycobacterium smegmatis*.

2. *M. avium* and *M. phlei* have nicotinamidase and asparaginase, respectively, and *M. smegmatis* contains strong nicotinamidase, asparaginase, and acetamidase.

3. *M. avium* and *M. smegmatis* have nicotinotransferase, and acetotransferase. *M. phlei* exhibits no appreciable activity of transferase for nicotinamide, benzamide. L-asparagine and acetamide, but slight activity L-glutamine.

4. The enzymatic transfer of acetyl group from acetamide to hydroxylamine is also studied with the extracts from *M. smegmatis*.

It is a pleasure for the author to express his gratitude to Prof. S. Akabori of Osaka University for his continuous guidance throughout this series of studies. His thanks are also due to Prof. T. Sasakawa for his kind help and interest, and to Messrs. K. Suzuki and S. Satoh for their technical assistance.

REFERENCE

(*1*) Kirchheimer, W. F., and Whittaker, C. K., *Am. Rev. Tuberc.*, **70**, 920 (1954)

(*2*) Halpern, Y. S., and Grossowicz, N., *Biochem. J.*, **65**, 716 (1957)

(*3*) Kimura, T., *J. Biochem.*, **46**, 973 (1959)

(*4*) Grossowicz, N., and Halpern, Y. S., *J. Biol. Chem.*, **228**, 643 (1957)

(5) Kimura, T., *J. Biochem.*, **46**, 97, 1133 (1959)
(6) Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945)
(7) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

BEHAVIOUR OF RIBONUCLEASES T_1 AND T_2 TOWARDS RIBO-APYRIMIDINIC ACIDS*

By SHOSUKE TAKEMURA and MASAZUMI MIYAZAKI

(*From the Department of Chemistry, Faculty of Science, Nagoya University, Nagoya*)

(Received for publication, January 26, 1959)

In a previous paper (*2*), it has been shown that hydrazinolysis of ribonucleic acid gives a nondialysable product free from pyrimidines, named ribo-apyrimidinic acid. A study on the behaviour of pancreatic ribonuclease towards the apyrimidinic acid has proved the importance of the pyrimidines in ribonucleic acid for the action of this enzyme (*3*). Hence, it appeared of interest to investigate the action of other ribonucleases on the apyrimidinic acid.

Ribonucleases T_1 and T_2 have been isolated from Takadiastase and purified by Sato and Egami (*4*). The specificity of these enzymes was quite different from that of pancreatic ribonuclease, that is they hydrolyse internucleotide linkages between the purine nucleoside 3′-phosphoryl groups and the 5′-hydroxyl groups of the adjacent nucleotides (*5*, *6*). The present paper deals with the behaviour of ribonucleases T_1 and T_2 towards the ribo-apyrimidinic acids.

EXPERIMENTAL

Substrates—Commercial yeast ribonucleic acid (Schwarz Laboratories Inc.) was purified by Sevag's method (*7*), dialysed against frequent changes of distilled water and lyophilised. Ribo-apyrimidinic acids, Preparations 1 and 2, were described in a previous paper (*2*), where they were listed as RAPYA I and IV respectively. Preparation 1 contains about 85 per cent of cytosine and about 70 per cent of uracil present in its parent yeast ribonucleic acid, but Preparation 2 is almost free from the pyrimidines.

Ribonucleases T_1 and T_2—These enzymes were prepared by Sato *et al.* of our laboratory (*4*) and are zone electrophoretically homogeneous.

Action of the Enzymes on the Ribo-apyrimidinic Acids—Ribonuclease T_1 (60 μg.) was incubated with 60 mg. portions of yeast ribonucleic acid and of the ribo-apyrimidinic acids (Preparation 1 and 2) in 4 ml. of 0.1 *M* tris(hydroxymethyl)amino methane buffer (pH 7.6) at 37°. An appropriate amount of ribonuclease T_2 was incubated with each of the nucleic acid and Preparation 2 (7 mg.) in 0.1 *M* acetate buffer (pH 4.5) at

* This study was presented at the 4th International Congress of Biochemistry in Wien, September 1-6, 1958 (*1*), and at the 7th Symposium on Nucleic Acids in Nagoya, February 1-2, 1958.

37°. Aliquots (1 volume) were added to acid—uranyl reagent (0.2 volume) which was the same as described in a previous paper (*3*). The acid soluble hydrolysis products served for the determination of phosphorus and plotted as per cent of total phosphorus, as shown in Fig. 1.

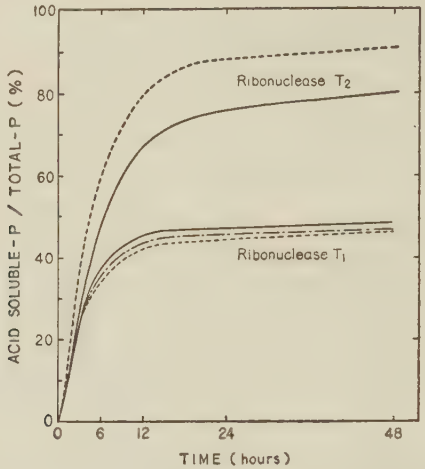


Fig. 1. Action of ribonucleases T_1 and T_2 on yeast ribonucleic acid and on its ribo-apyrimidinic acids. —— ribo-ayprimidinic acid, Preparation 2; ——— ribo-apyrimidinic acid, Preparation 1; ---- ribo-nucleic acid.

RESULTS AND DISCUSSION

It can be seen in Fig. 1. that ribonuclease T_1 equally hydrolyses both the ribo-apyrimidinic acids and the nucleic acid. The ribo-apyrimidinic acid is also considerably hydrolysed by ribonuclease T_2. These results are quite contrary to that obtained by the action of pancreatic ribonuclease on the ribo-apyrimidinic acids (*3*), that is, the presence in ribonucleic acid of the pyrimidines is not essential for the catalytic activities of ribonucleases T_1 and T_2. Ribonuclease T_1 catalyses the cleavage of the internucleotide bonds between the guanosine 3′-phosphoryl groups and the 5′-hydroxyl groups of the adjacent nucleotides (*5*, *6*). The T_2 enzyme is specific to the adenosine 3′-phosphoryl esters (*8*). On account of these high specificities, it is possible that the presence in ribonucleic acid of the purines is required for the action of these enzymes. To confirm this point, it is desirable to find a route by which the purines are removed from ribonucleic acid.

SUMMARY

1. The behaviour of ribonucleases T_1 and T_2 towards ribo-apyrimidinic acids was studied.

2. Both of these enzymes did not required the presence in ribonucleic

acid of the pyrimidines for their catalytic activities.

The authors thanks Prof. F. Egami for his close interest. The expense of this study was defrayed in part by a grant to Prof. F. Egami from the Ministry of Education.

REFERENCES

(*1*) Takemura, S., *IV. Intern. Kongr. Biochem., Zusammenfassungen*, Wien, p 34 (1958)
(*2*) Takemura, S., *J. Biochem.*, **44**, 321 (1957); *Bull. Chem. Soc. Japan*, in press
(*3*) Takemura, S., Takagi, M., Miyazaki, M., and Egami, F., *J. Biochem.*, in press
(*4*) Sato, K., and Egami, F., *J. Biochem.*, **44**, 753 (1957)
(*5*) Sato, K., and Egami, F., *Compt. rend. soc. biol.*, **151**, 1792 (1957)
(*6*) Sato-Asano, K., *J. Biochem.*, **46**, 31 (1959)
(*7*) Sevag, M. G., *Biochem. Z.*, **273**, 419 (1934)
(*8*) Naoi-Tada, M., Sato-Asano, K., and Egami, F., *J. Biochem.*, **46**, 757 (1959)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

BEHAVIOUR OF PANCREATIC DEOXYRIBONUCLEASE TOWARDS DEOXYRIBO-APYRIMIDINIC ACIDS*

By SHOSUKE TAKEMURA

(*From the Department of Chemistry, Faculty of Science, Nagoya University, Nagoya*)

(Received for publication, January 26, 1959)

The correlation between the action of pancreatic deoxyribonuclease (deoxyribonuclease I), which is a highly specific phosphodiesterase (*2*, *3*), and the structure of its substrate is not yet clear. Tamm *et al.* (*4*) reported that partial or total removal of the purines from deoxyribonucleic acid greatly diminished the catalytic effects of this enzyme. Hitherto, however, it has been impossible to investigate the participation of the pyrimidines in the action of the enzyme, because of unavailability of a derivative of deoxyribonucleic acid free from the pyrimidines.

In a previous paper from this laboratory (*5*), it has been shown that hydrazinolysis of deoxyribonucleic acid affords a nondialysable product devoid of the pyrimidines, named deoxyribo-apyrimidinic acid. By controlling the reaction conditions the acids containing greater or lesser amounts of the pyrimidines could be obtained.

The present paper deals with the action of pancreatic deoxyribonuclease on the deoxyribo-apyrimidinic acids of different pyrimidine content and shows the pyrimidines in deoxyribonucleic acid closely participate in the activity of this enzyme.

EXPERIMENTAL

Substrates—Herring sperm deoxyribonucleic acid**, deoxyribo-apyrimidinic acids of different pyrimidine content, Preparations 1, 2 and 3, and apurinic acid were employed. The properties and the composition of these substrates were described in a previous publication (*5*), where the deoxyribo-apyrimidinic acids, Preparations 1, 2 and 3, were listed as DAPYA I, II and IV, For convenience, the pyrimidine contents of these substrates as per cent, of that present in the parent deoxyribonucleic acids are shown in Table I.

Pancreatic Deoxyribonuclease (*deoxyribonuclease I*)—A commercial crystalline specimen, Worthington Biochemical Sales Company, was used.

* This study was presented at the 4th International Congress of Biochemistry in Wien, September 1-6, 1958 (*1*), and at the 7th Symposium on Nucleic Acids in Nagoya, February 1-2, 1958.

** The author is greatly indebted to Prof. I. Watanabe, University of Tokyo, for a supply of the deoxyribonucleic acid.

Method—0.3 per cent solutions of the substrates in 0.1 *M* phosphate buffer (pH 6.5) or tris(hydroxymethyl)amino methane buffer (pH 7.2) were incubated with deoxyribonuclease I in the presence of 0.02 *M* magnesium sulfate as an activator (2, 6–10). In control experiments the enzyme was omitted. Aliquots (1 volume) were added to acid-uranyl reagent (0.2 volume) which was the same as described in previous paper (*11*). The acid soluble hydrolysis products in the supernatants were estimated by the colour intensity of the reaction with diphenylamine (*12*) and expressed as per cent of total intensity.

TABLE I

Base Content in the Substrates[1]

Substrate[1]	Adenine (%)	Guanine (%)	Cytosine (%)	Thymine (%)
DAPYA[2], Preparation 1	100	100	5.7	70.4
2	100	100	3.2	29.6
3	100	100	1.4	15.2
Apurinic acid	2.1	4.4	100	100

1) These values are shown as per cent of each bases in the parent deoxyribonucleic acids.

2) DAPYA=deoxyribo-apyrimidinic acid.

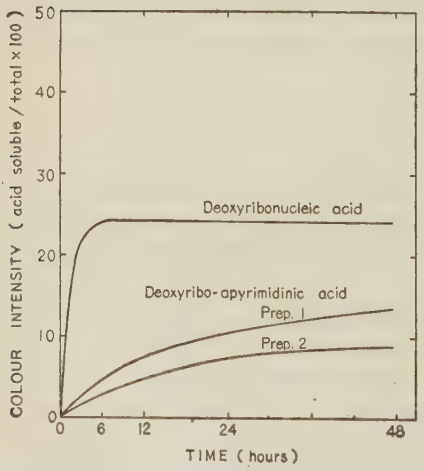


Fig. 1. Action of pancreatic deoxyribonuclease on herring sperm deoxyribo-apyrimidinic acids of different pyrimidine content. Enzyme/substrate =1/60, pH 6.5, 37°, in the presence of 0.02 *M* Mg^{++}.

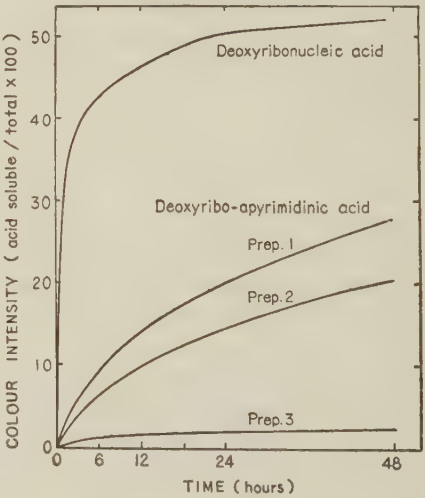


FIG. 2. Action of pancreatic deoxyribonuclease on herring sperm deoxyribo-apyrimidinic acids of different pyrimidine content. Enzyme/substrate =1/20, pH 7.2, 37° in the presence of 0.02 *M* Mg^{++}.

Action of the Enzyme on the Apyrimidinic Acids—(a) with an enzyme substrate ratio of 1 to 60—Preparations 1 and 2 were used. The experiments were carried out in phosphate buffer (0.1 *M*, pH 6.5) at 37°. The results are shown in Fig. 1. (b) with an enzyme substrate ratio of 1 to 20—Preparations 1, 2 and 3, and apurinic acid were used. The experiments were carried out in Tris buffer (0.1 *M*, pH 7.2) at 37° and the results are presented in Fig. 2.

Results—The substrates in which the pyrimidines were partially removed from the nucleic acid were resistant to the action of the enzyme, and as the pyrimidine content of the substrates decreased the rate of hydrolysis of the substrates was reduced. Only very small amounts of acid soluble products were produced by the enzyme from Preparation 3 which was free from the cytosine but contained about 15 per cent of the thymine present in its parent nucleic acid. Like Preparation 3, apurinic acid was only slightly hydrolysed.

DISCUSSION

There have been several attempts to identify oligonucleotides amongst the degradation products present in deoxyribonuclease I digests of deoxyribonucleic acids (*13–19*). Whereas the correlation between the action of the enzyme and the structure of its substrate is not yet clear. The present data show the close relation of pyrimidines in deoxyribonucleic acid to the catalytic activity of the enzyme. The removal of the pyrimidines from the nucleic acid depresses the activity of this enzyme, thus the rate of hydrolysis of a deoxyribo-apyrimidinic acid of low pyrimidine content is less than that of one with a higher pyrimidine content. Deoxyribo-apyrimidinic acid, Preparation 3, is practically unhydrolysed to form acid soluble products, although it contains about 15 per cent of the thymine present in its parent deoxyribonucleic acid. This enzyme digests deoxyribonucleic acid to oligonucleotides, in part to dinucleotides (roughly 16 per cent), but gives only small amounts (*ca.* 1 per cent) of mononucleotides (*13*, *14*). Therefore, it cannot be denied that Preparation 3 may be hydrolysed to oligonucleotides which are precipitated by the acid-uranyl reagent. The importance of pyrimidines was also observed in the action of pancreatic ribonuclease (ribonuclease I) and of its active components (ribonucleases A and B) on ribo-apyrimidinic acid (*11*). It seems very probable that the presence of the pyrimidines in deoxyribonucleic acid is necessary for the action of deoxyribonuclease I, although absolute proof of this has yet to be obtained.

The experiments of Tamm et al. on the action of deoxyribonuclease I on apurinic acid lead them to propose that the principal requirement for the maximal effect of this enzyme, activated by magnesium, was the presence in deoxyribonucleic acid of the full complement of purines (*4*). They also expected that the preservation of the pyrimidines was equally necessary. The present data give support to their view. That is, it seems probable that the presence in deoxyribonucleic acid not only of the purines but also of the pyrimidines is necessary for the full catalytic activity of this enzyme.

SUMMARY

1. The bahaviour of pancreatic deoxyribonuclease (deoxyribonuclease I) towards deoxyribo-apyrimidinic acids of different pyrimidine content was studied.

2. As the pyrimidine content in the deoxyribo-apyrimidinc acids was decreased, the rate of hydrolysis of these substrates by the enzyme was reduced.

3. Together with the experiments on apurinic acid by Tamm *et al.*, it can be said that the presence in deoxyribonucleic acid not only of the purines but also of the pyrimidines is necessary for the full catalytic activity of the enzyme.

The author thanks Prof. F. Egami for his close interest. The expense of this study was defrayed in part by a grant to Prof. F. Egami from the Ministry of Education.

REFERENCES

(*1*) Takemura, S., *IV. Intern. Kongr. Biochem., Zusammenfassungen*, Wien, p 34 (1958)
(*2*) Laskowski, M., *Arch. Biochem.*, **11**, 41 (1946)
(*3*) McCarty, M., *J. Gen. Physiol.*, **32**, 39 (1948)
(*4*) Tamm, C., Shapiro, H. S., and Chargaff, E., *J. Biol. Chem.*, **199**, 313 (1952)
(*5*) Takemura, S., *Biochim. et Biophys. Acta*, **29**, 447 (1958); *Bull. Chem. Soc. Japan*, in press
(*6*) Laskowski, M., and Seidel, M. K., *Arch. Biochem.*, **7**, 465 (1945)
(*7*) McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946)
(*8*) Overend, W. G., and Webb, M., *Research*, **2**, 99 (1949)
(*9*) Kunitz, M., *J. Gen. Physiol.*, **33**, 349 (1950)
(*10*) Miyaji, T., and Greenstein, J. P., *Arch. Biochem. Biophys.*, **32**, 414 (1951)
(*11*) Takemura, S., Takagi, M., Miyazaki, M., and Egami, F., *J. Biochem.*, in press
(*12*) Dische, Z., *Mikrochem.*, **8**, 4 (1930); Seibert, F. B., *J. Biol. Chem.*, **133**, 593 (1940)
(*13*) Potter, J. L., Brown, K. D., and Laskowski, M., *Biochim. et Biophys. Acta*, **9**, 150 (1952)
(*14*) Sinsheimer, R. L., *J. Biol. Chem.*, **208**, 445 (1954)
(*15*) Gordon, A. H., and Reichard, P., *Biochem. J.*, **48**, 569 (1951)
(*16*) Sinsheimer, R. L., and Koerner, J. F., *Science*, **114**, 42 (1951)
(*17*) Smith, J. D., and Markham, R., *Biochim. et Biophys Acta*, **8**, 350 (1952)
(*18*) Sinsheimer, R. L., *J. Biol. Chem.*, **215**, 579 (1955)
(*19*) Privat de Garilhe, H., Cunningham, L., Laurila, U., and Laskowski, M., *J. Biol. Chem.*, **224**, 751 (1957)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

TERMINAL OXIDATION SYSTEM IN BACTERIA

V. PRELIMINARY STUDY ON PHYSIOLOGICAL FUNCTION OF THE RESPIRATORY COMPONENTS OF PSEUDOMONAS AERUGINOSA

By TATEO YAMANAKA

(From the Department of Biology, Faculty of Science, University of Osaka, Osaka)

(Received for publication, March 2, 1959)

During the earlier work of Horio (*1*, *2*), Okunuki *et al.* (*3*), and Higashi (*4*), some respiratory components of *Pseudomonas aeruginosa* were solubilized without the aid of any detergent and were separately purified: *Pseudomonas* (P-) cytochrome$_{554}$, P-cytochrome$_{551}$, P-blue protein, P-cytochrome oxidase and P-hydroquinone oxidase. Using a highly purified preparation, P-cytochrome oxidase has been found to contain so-called cytochrome a_2 (*3*). P-blue protein (*2*) and P-cytochrome$_{551}$ (*5*) have been prepared in crystalline form. Both of the oxidized components are reduced by lactate in the presence of yeast lactic dehydrogenase (containing cytochrome b_2) (*6–10*). The reduced components are oxidized by oxygen in the presence of P-cytochrome oxidase, the oxidation being strongly inhibited by carbon monoxide and cyanide (*2*, *3*). Lactate-oxidation in living cells of *Pseudomonas aeruginosa* was found to be strongly inhibited by the same respiratory inhibitors (*2*, *4*). Hereby, it is sure that in the terminal electron transferring system of the microorganism these respiratory components are functional between a substrate and oxygen (oxygen respiration) in a similar manner to that in animal mitochondria (*11–15*). Besides these respiratory components, which have been separately purified, this bacterium appears on observation with a hand-spectroscope to have two more cytochromes. These might be cytochromes similar to the so-called cytochrome b_1 and cytochrome a_1 (*1*).

It is well known that *Pseudomonas aeruginosa* is a denitrifying bacterium and can grow under anaerobic conditions in the presence of nitrate as well as in its absence under aerobic conditions. Using a strain of *Pseudomonas aeruginosa*, Verhoeven and Takeda (*16*) showed that a "cytochrome c" type pigment with an α-absorption peak at 552 mμ might be functional in this nitrate reduction (nitrate respiration). Moreover, Taniguchi, Sato and Egami (*17*) reported that cytochrome b_1 which shows an α-absroption peak around 560 mμ has an important role in the nitrate respiration of *Escherichia coli*.

Horio *et al.* (*2*, *3*) has described a possible relationship of the four kinds of respiratory components separately purified from *Pseudomonas aeruginosa* on

the basis of their properties. The present paper describes some critical experiments to confirm the physiological function of the respiratory components present in the living cells. Two different samples prepared from *Pseudomonas aeruginosa* have been used. These appear to maintain a more organized interaction of the respiratory components and enzymes than the separately purified components. These preparations were: a sonicated preparation of the cells and a crude extract of the acetone-treated cells. A possible scheme for oxygen and nitrate respiration of *Pseudomonas aeruginosa* is presented.

MATERIALS AND METHODS

The same strain of *Pseudomonas aeruginosa* was used through this series of experiments (*1–5*). Under aerobic conditions, this bacterium can grow in the absence of nitrate. With a handspectroscope it can be seen that grown cells show absorption bands essentially same as those of the cells grown in a nitrate-containing medium under anaerobic conditions. In this study, the bacterium was grown on a common bouillon-peptone-agar medium (pH 7) in air for one day at 37°. The grown cells were washed four times with distilled water in a homogenizer and centrifuged ($10{,}000 \times g$ for 10 minutes) after each wash. The washed cells were suspended in distilled water. In some experiments, one per cent sodium lactate or succinate was added to the standard medium.

The cell suspension (about 50 mg. dry weight per ml.) was disintegrated in a Raytheon Sonic Oscillator (9 KC.) cooled by ice water for a fixed time, and then centrifuged at $10{,}000 \times g$ for 20 minutes. The resulting supernatant was once more centrifuged in the same way. The recentrifuged supernatant was used as the cell-free extract.

From the grown cells an acetone powder was made at −5° to 0° by the usual method and dried overnight at room temperature (10°–15°) over $CaCl_2$ in a desiccator. The dried acetone powder was suspended in 0.1 *M* sodium citrate (pH 7.6) and heated at about 40° for 10 minutes. It was immediately chilled in an ice-water bath. The chilled suspension was centrifuged at $20{,}000 \times g$ for one hour, and the resulting supernatant was fractionated by cold acetone. The fraction precipitating at between 30 and 70 per cent acetone was collected. It was well drained, dissolved in distilled water and centrifuged at $10{,}000 \times g$ for 15 minutes. The supernatant was not completely clear. Solid ammonium sulphate was added to the supernatant to 70 per cent-saturation, and the mixture was then centrifuged. The precipitate was dissolved in 30 per cent-saturated ammonium sulphate and centrifuged. The resulting supernatant was clear and was used as the lactic dehydrogenase preparation.

The cell-free extract of the cells described above was dialysed overnight against tap water in a refrigerator and then precipitated by the addition of solid ammonium sulphate at near saturation. The precipitate was suspended in distilled water and the suspension was centrifuged at $10{,}000 \times g$ for 20 minutes. The supernatant was used as the succinic dehydrogenase preparation.

P-cytochrome$_{551}$ was purified to about 80 per cent purity according to the method of Horio (*1*).

Oxygen consumption was measured by a Warburg manometric apparatus at 30°.

The cells grown aerobically under the culture conditions described above could under anaerobic conditions reduce nitrate when succinate or lactate was added, but nitrite was not reduced. However cells grown anaerobically in the presence of nitrate could reduce nitrite as well as nitrate. Therefore with the anaerobically grown cells nitrite may not be estimated as a metabolic intermediate of nitrate. The nitrite-reducing enzyme of this

bacterium appeared to be produced adaptively in the presence of nitrate under anaerobic conditions. When cells grown aerobically were used, the nitrate-reducing activity of the cells could therefore be measured by determination of the nitrite formed during a certain time. If nitrate was added, the cells grown aerobically reduced nitrate to nitrite at an almost linear rate at 30° during 30 minutes. To measure nitrate-reduction, Thunberg tubes were used: the main chamber contained 1.0 ml. of 0.2 *M* sodium phosphate buffer of pH 7.0, 0.1 ml. of 0.2 *M* sodium nitrate and 0.2 ml. of 0.2 *M* substrate (sodium lactate or succinate, pH 7.0) or distilled water, and the side chamber contained 0.3 ml. of an enzyme preparation and 0.4 ml. of distilled water. After the tubes had been evacuated and filled with nitrogen gas, they were immersed in a water bath at 30°. After five minutes-preincubation, reactions were started by mixing the reaction components of both chambers. After ten minutes, 1.0 ml. of the reaction mixture was pipetted out into 5.0 ml. of 10 per cent trichloroacetic acid. The mixture was filtered. Nitrite present in the filtrate was determined according to the method of Treadwell (*18*): to 3.0 ml. of the filtrate, 0.5 ml. of 1 per cent sulfanilamide and then 0.5 ml. of 0.02 per cent *N*-(1-naphthyl) ethylenediamine hydrochloride were added. After about 30 minutes, the red colour developed was measured at 540 mμ.

The optical density was measured with a Shimadzu photoelectric spectrophotometer, type QB-50. The absorption spectrum of a turbid solution such as that of the cell suspension or cell-free extract was measured using an opal glass according to the method of Calvin and Shibata (*19*).

RESULTS

With Lactic and Succinic Dehydrogenase Preparations—The lactic dehydrogenase preparation could reduce methylene blue and P-cytochrome$_{551}$ in the presence of lactate without the addition of diphosphopyridine nucleotide (DPN) but not in the presence of succinate. The optimal pH's were, about 6.4 for the methylene blue reduction and about 6.0 for the P-cytochrome$_{551}$ reduction, as shown in Fig. 1. The enzyme preparation contained P-cytochrome$_{554}$ and P-cytochrome$_{551}$: the former was always present in larger amounts than the latter. As in the case of the reductions of added P-cytochrome$_{551}$ and methylene blue, both the cytochromes present in the enzyme preparation were immediately reduced if lactate was added. The enzymic reduction of methylene blue and the cytochromes were accelerated by the addition of DPN. DPNH could replace lactate. The lactic dehydrogenase preparation did not appear spectroscopically to be contaminated with other substances having absorption spectra at a visible wavelength other than P-cytochrome$_{554}$ and P-cytochrome$_{551}$. At this step of purification this enzyme preparation was much cruder, and based on the results mentioned above, contained at least a DPNH-essential lactic dehydrogenase, DPNH-cytochrome reductase and DPN. Since the normal redox-potential of P-cytochrome$_{554}$ is about 25 mv. lower than that of P-cytochrome$_{551}$ (*2*), it seemed likely that the reductase present in the enzyme preparation might be P-cytochrome$_{554}$ reductase. However the enzyme has not yet been purified further.

Taking into consideration the purification procedures, the succinic dehydrogenase preparation probably contained both water-soluble and -insoluble

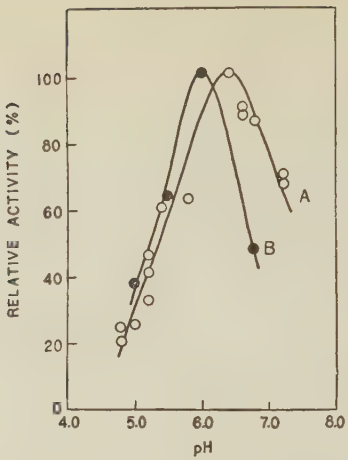


FIG. 1. Influences of pH on reductions of methylene blue and P-cytochrome$_{551}$ by lactate in the presence of lactic dehydrogenase. In the case of methylene blue-reduction, Thunberg tubes were used with the following components: main chamber, 2.0 ml. of 0.2 *M* phosphate buffer of various pH's, 0.5 ml. of 0.1 *M* sodium lactate, 0.5 ml. of 5×10^{-5} *M* methylene blue and 0.2 ml. of 0.5 *M* semicarbazide. Side chamber, 0.5 ml. of lactic dehydrogenase. Reactions were carried out at 30°, and reduction of methylene blue at pH 6.4 required ten minutes. Relative activity for the methylene blue-reduction was simply calculated with minutes required for the complete reduction of methylene blue. Reduction of P-cytochrome$_{551}$ was measured by the increment of optical density at 551 mμ, the α-absorption maximum of the cytochrome. The reaction was carried out at room temperature (23°) with the following components: 2.0 ml. of 0.2 *M* phosphate buffer of various pH's 0.5 ml. of 0.1 *M* sodium lactate, 0.5 ml. of P-cytochrome$_{551}$ and 0.5 ml. of lactic dehydrogenase preparation. The reaction was started by addition of the enzyme, and readings were made every 30 seconds after mixing the components. With solid sodium dithionite added in excess to the reaction mixture at the end of the reaction, the increment in extinction at 551 mμ was about 0.06. Calculations of relative activity for the P-cytochrome$_{551}$-reduction are based on values for the initial two minutes reaction which proceeds linearly. Curve A, reduction of methylene blue; curve B, reduction of P-cytochrome$_{551}$.

cellular substances. The bacterium has been found to contain a cytochrome with an α-absorption peak at around 560 mμ, like the so-called cytochrome b_1. This pigment can be repeatly oxidized and reduced like other cytochromes (*1*, *2*). So far, the pigment has not yet been solubilized without the aid of sodium cholate, remaining in the cellular fragments at a tightly bound

state. Using a handspectroscope it was found that when succinate was added, the P-cytochrome$_{554}$ and P-cytochrome$_{551}$ in the succinic dehydrogenase preparation were strongly reduced, and only in a concentrated enzyme sample, could an absorption band at around 560 mμ be seen. Pappenheimer and Hendee (*20*) have found that cytochrome b_1 has an intimate relationship with succinic dehydrogenase like the cytochrome b in animal mitochondria. Therefore, the absorption band around 560 mμ might be that of a cytochrome b_1 type pigment. When succinate was added, the succinic dehydrogenase preparation could reduce added P-cytochrome$_{551}$.

With Resting Cells—Resting cells of *Pseudomonas aeruginosa* could rapidly oxidize succinate and lactate. In a cell suspension, the oxidation of lactate was always more rapid than that of succinate, though the oxidation of both substrates varied greatly due to differences of the culture conditions, as shown

TABLE I

Summary of Oxygen-consuming and N trate-reducing Activities

Sample	Activity	Oxygen consumed and nitrate reduced (μ *atom or* μ *mole/mg. dry weight/hour*)		
		In the presence of		(Ratio) Lactate/succinate
		lactate	succinate	
Resting cells	Oxygen consumed	5.7	5.2	1.1
	Nitrate reduced	0.24	0.38	0.63
Resting cells (+lactate)*	Oxygen consumed	2.3	1.8	1.3
	Nitrate reduced	0.23	0.37	0.65
Resting cells (+succinate)*	Oxygen consumed	2.1	0.89	2.4
	Nitrate reduced	0.15	0.22	0.68
Cell-free extract	Oxygen consumed	1.3	11	0.12
	Nitrate reduced	0.31	0.50	0.62

* With cells grown in a medium containing lactate or succinate.

in Table I. Cells grown in a lactate- or succinate-medium were much more viscous than cells grown in a standard medium. This might be due to the production of many viscous substances so that the oxidation of the substrates by the cells grown in the substrate-containing medium were much slower than oxidation by cells grown in the standard medium. In contrast to the effect on oxidation, it was found that cytochromes with absorption bands from 550 to 560 mμ which were present in the cells grown in the substrate-containing medium, were almost of the same concentrations as those in cells grown in the standard medium (Fig. 2).

As shown in Table I when nitrate was added as the final electron acceptor either *in vacuo* or under nitrogen, the resting cells could oxidize succinate and lactate by reducing the nitrate to nitrite. Unlike observations on cell oxidation, it was found that with cells grown in various media, nitrate reduction by succinate was always faster than that by lactate. The ratios of the rate of nitrate reduction by lactate to that by succinate were almost the same.

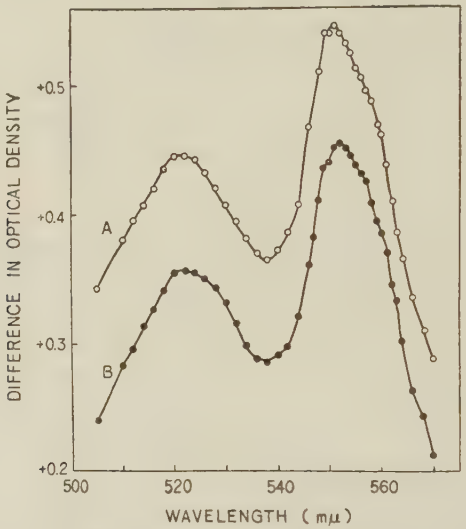


FIG. 2. Differential spectra of cells grown in the standard medium and in the succinate medium. Grown cells were well-suspended in distilled water. The suspension contained approximately 10 mg. dry weight of cells per ml. Optical densities were measured by the difference between cell suspensions previously reduced by sodium dithionite and cell suspensions previously oxidized by potassium ferricyanide. Curve A, cells grown in the standard medium; Curve B, cells grown in the succinate medium.

Even after several washings and long time-aeration cytochromes with absorption bands from 550 to 560 mμ were about one-third in the reduced form in the cells. When nitrate was added to these cells, absorption bands around 560 mμ and 530 mμ notably decreased, as shown in Fig. 3. These bands could be the α- and β-absorption peaks of a cytochrome. Hereafter, this cytochrome will be tentatively called P-cytochrome$_{(560)}$. The parentheses are used because the cytochrome has not yet been finely examined in its pure form. The oxidation of P-cytochrome$_{(560)}$ by nitrate indicates that this cytochrome$_{(560)}$ plays an important role in the nitrate respiration of *Pseudomonas aeruginosa.*

When the cells were grown in the presence of nitrate under anaerobic conditions, the ratio of the amount of P-cytohrome$_{(560)}$ to P-cytochrome$_{554}$

and P-cytochrome$_{551}$ was higher than that in the cells grown aerobically.

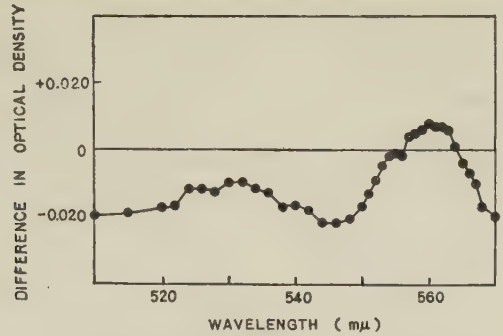


FIG. 3. Differential spectrum of cells in the stationary state in the presence and absence of nitrate. Cells grown in the standard medium were suspended in distilled water (approximately 5 mg. of cells in dry weight per ml.). Optical densities were measured by comparing the cell suspension with a suspension previously oxidized by potassium ferricyanide. Sodium nitrate was added to the cell suspension to approximately 0.01 *M* final concentration. The differential extinction = (stationary extinction of cells in the absence of nitrate) − (stationary extinction of cells in the presence of nitrate).

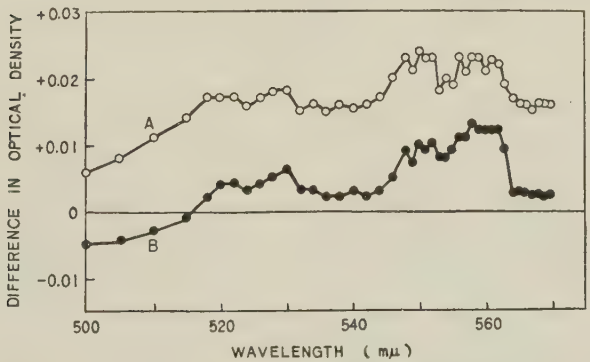


FIG. 4. Differential spectra of cells in the presence and absence of succinate and of lactate. The cells grown in the standard medium were suspended in 0.05 *M* phosphate buffer of pH 7.0 (approximately 10 mg. of cells in dry weight per ml.). Optical densities were read with the following components against the same components previously oxidized by potassium ferricyanide: 3.0 ml. of cell suspension, 0.5 ml. of distilled water, 1.0 *M* sodium succinate or 1.0 *M* sodium lactate, and 1.0 ml. of distilled water. Curve A, (+lactate)−(+none); Curve B, (+succinate)−(+none). When sodium dithionite was added in excess, the differential spectrum was almost same as Curve B.

When succinate and lactate were added to well washed cells which had been aerated for a long time, the cytochromes present in the cells were strongly reduced. As shown in Fig. 4, the cytochromes with absorption band from 550 to 560 mμ (the α-absorption bands of P-cytochrome$_{551}$, P-cytochrome$_{554}$

and P-cytochrome$_{(560)}$) were reduced most by the addition of succinate as well as by the addition of excess sodium dithionite, while a small amount of P-cytochrome$_{(560)}$ still remained in its oxidized form after the addittion of lactate. This implies that these cytohromes play important roles in the oxidations of the substrates.

With a Cell-Free Extract—In contrast to the oxidation of succinate and lactate by the resting cells, succinate oxidation by a cell-free extract was much faster than lactate oxidation, as shown in Table I. On the other hand, the ratio of the rate of nitrate reduction by lactate to that by succinate was almost the same as for resting cells.

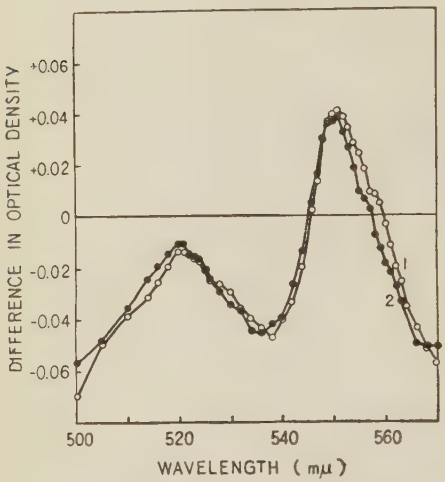


FIG. 5. Reduction of cytochromes present in a cell-free extract by succinate and reoxidation of the reduced cytochromes by addition of nitrate. Optical densities were read with the following components: 3.0 ml. of the cell-free extract (8.3 mg. in dry weight per ml.), 0.5 ml. of 0.2 *M* phosphate buffer of pH 7.0 and 0.5 ml. of 0.5 *M* sodium succinate. When nitrate was added, 0.25 ml. of 1.0 *M* sodium nitrate was added after the cell-free extract had been reduced by addition of 0.25 ml. of 1.0 *M* succinate. Curve 1, succinate; Curve 2, succinate and then nitrate.

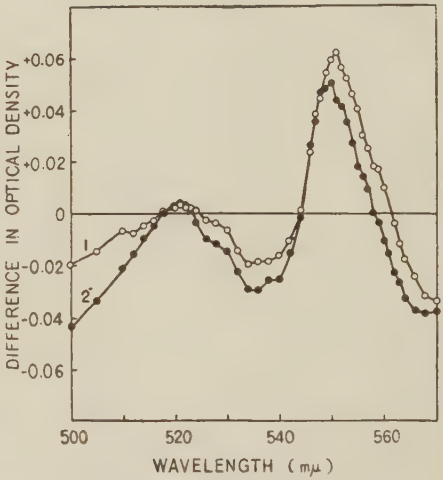


FIG. 6. Reduction of cytochromes by lactate in a cell-free extract.

Experimental conditions as for Fig. 5 except that sodium lactate was used in place of succinate.

Curve 1, lactate.

Curve 2, lactate and then nitrate.

The cytochromes present in the cell-free extract unlike in resting cells were present in their oxidized forms. When succinate and lactate were added to the extract, the cytochromes were immediately reduced to an equilibrium state. As with resting cells, the P-cytochrome$_{551}$ and P-cytochrome$_{554}$ present in the extract were reduced to almost the same extent by the addition of succinate or lactate. P-cytochrome$_{(560)}$ was reduced further by succinate than by lactate, as shown in Figs. 5 and 6. This indicates P-cytochrome$_{(560)}$ has a more intimate connection with succinate dehydrogenation than the other two

cytochromes, and that P-cytochrome$_{(560)}$ might not have a direct relation to lactate dehydrogenation, reinforced by the results obtained with the lactate dehydrogenase preparation. When nitrate was added to the cell-free extract previously supplemented with succinate and lactate, the cytochromes in the extract were partially oxidized. As shown in Fig. 7, after previous supplementation with lactate, P-cytochrome$_{551}$, P-cytochrome$_{554}$ and P-cytochrome$_{(560)}$

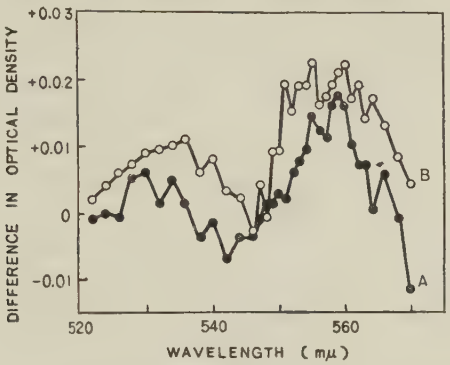


FIG. 7. Differential spectra of cell-free extract reduced by succinate and lactate in the presence and absence of nitrate.
Curve A; Curve 1—Curve 2 in Fig. 5.
Curve B; Curve 1—Curve 2 in Fig. 6.

were oxidized by the addition of nitrate. After previous supplementation with succinate, P-cytochrome$_{554}$ and P-cytochrome$_{(560)}$ were oxidized but P-cytochrome$_{551}$ was hardly oxidized. This indicates that these three cytochromes are directly or indirectly functional in the nitrate reduction as well as in the succinate and lactate oxidation.

The lactate-oxidizing activity of the cell-free extract was altered by the duration of sonic treatment of the cells: with longer durations of sonication, the concentration of the three kinds of cytochromes and the lactate- and succinate-oxidizing activities of the extract increased. But after prolonged sonication the lactate-oxidizing activity was lost more than the succinate-oxidizing activity, while the concentration of cytochromes was increased. The more P-cytochrome$_{551}$ enzymatically reduced in the presence of lactate and of DPNH, the stronger was the lactate- and DPNH-oxidizing activity of the extract.

DISCUSSION

With the use of the opal glass-method (*19*), oxidation and reduction of the α-absorption bands of P-cytochrome$_{551}$, P-cytochrome$_{554}$ and P-cytochrome$_{(560)}$ present in the living cells of *Pseudomonas aeruginosa* are spectrophotometrically measurable. When succinate or lactate is added to the cells in the stationary state, the three cytochromes are reduced. When nitrate is added to grown cells, of the three cytochromes which are approximately one-third in their

reduced forms, only P-cytochrome$_{(560)}$ is notably oxidized. The cell-free extract also contains the three cytochromes. When succinate or lactate is added to the cell-free extract, the three cytochromes present in the extract are reduced. The components previously reduced by the addition of lactate and of succinate are partially oxidized again when nitrate is added. After previous reduction by succinate, P-cytochrome$_{(560)}$ and P-cytochrome$_{554}$ are partially reoxidized but cytochrome$_{551}$ is hardly affected. After previous reduction by lactate, all three cytochromes can be partially reoxidized. Since P-cytochrome$_{(560)}$ in all cases can be oxidized by the addition of nitrate, it is sure that this cytochrome has an intimate relation to the nitrate respiration of *Pseudomonas aeruginosa* as well as to succinate and lactate oxidation. The differences in kinds and extents of cytochromes oxidized by the additions of nitrate, lactate and succinate are for the most part definitely caused by the variations in the activities of enzymes to dehydrogenate succinate and lactate. Even when, in aerobic respiration, the succinate-oxidizing activity of the cells is weaker than the lactate-oxidizing activity, succinate is always a better substrate in nitrate respiration than lactate. This implies that the three cytochromes are functional in both oxygen and nitrate respiration, having a state of dynamic equilibrium with each other, and that P-cytochrome$_{(560)}$ has a more intimate connection with nitrate respiration than the other two cytochromes. Therefore, it seems likely that P-cytochrome$_{(560)}$ has the lowest oxidation-reduction potential of the three cytochromes. This conclusion about the redox-potential of P-cytochrome$_{(560)}$ may be supported by previous findings of Horio (*1*, *2*) that the redox-potentials of P-cytochrome$_{554}$ and P-cytochrome$_{551}$ were approximately +225 mv. and +250 mv., respectively. From the experiments with the lactic dehydrogenase preparation, it appears that the electrons liberated from lactate are accepted by DPN, P-cytochrome$_{554}$ and then P-cytochrome$_{551}$. On the other hand, it seems likely that the electrons liberated from succinate are transferred first to P-cytochrome$_{(560)}$.

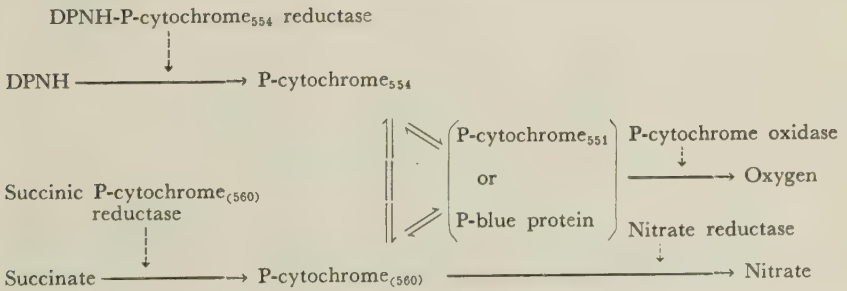
In the earlier work on crystalline cytochrome c's prepared from various sources by Okunuki *et al.* (*21-24*), it was demonstrated that cytochrome c like other enzymes has its own secondary structure in its protein-moiety and that the oxidation-reduction reaction in its heme-moiety is coupled with a certain change in this secondary structure. It is not known what function the coupled change plays in cellular respiration. *Pseudomonas aeruginosa* has another respiratory component, P-blue protein, which is not a cytochrome and whose normal redox-potential is about +300 mv. (*1*, *2*). Using the separately purified respiratory components, P-cytochrome$_{554}$, P-cytochrome$_{551}$, P-blue protein and a typical cytochrome c, it has been found that an electron can be smoothly transferred from a respiratory component in its reduced form to another component of a higher normal redox-potential, in its oxidized form, despite possible steric hinderance caused by the protein-moieties of the respiratory components (*2*). Therefore, it may be supposed that if any two respiratory components are in a state of equilibrium in their oxidation-reduction reaction, the equilibrium is mainly dependent on the difference between their

redox-potentials and, unlike in substrate-enzyme reactions, specificity in this reductant-oxidant reaction is not significant.

Although there is no evidence for oxidant-reductant specificity of the respiratory components in their oxidation-reduction reactions, there is some evidence that enzymes directly concerned with the terminal electron-transferring system have cytochrome-specificity as well as substrate-specificity. Bach, Dixon and Zerfas (*6*) purified yeast lactic dehydrogenase from baker's yeast, and Appleby and Morton (*7*) have succeeded in its crystallization. The enzyme contains one molecule of flavin mononucleotide, eight non-heme iron atoms and one heme iron atom per molecule (*25*). It can catalyse reduction of cytochrome c by lactate *via* the cytochrome b_2 in the enzyme. On the other hand, cytochrome b_2 has been crystallized from baker's yeast and from an enzyme preparation by Yamashita *et al.* (*8*): but like normal cytochromes, cytochrome b_2 has no enzymic activity, no flavin, and has the same absorption spectrum as that of the whole enzyme except in the ultraviolet region. The lactate-dehydrogenating enzyme appears to bind cytochrom b_2 specifically (*9*, *10*). Strittmatter and Velick (*26*) have purified DPNH-cytochrome b_5 reductase which can reduce cytochrome b_5 by DPNH but not cytochrome c. A similar reductase has been purified in a water-soluble state by Raw *et al.* (*27*, *28*), though this latter reductase may be tightly bound to cellular particles in its true functional state. Cytochrome-specificity has clearly demonstrated with cytochrome oxidase (*2*, *3*): P-cytochrome oxidase containing so-called cytochrome a_2 can rapidly oxidize P-cytochrome$_{551}$ but only oxidizes slowly P-cytochrome$_{554}$ and typical cytochrome c's crystallized from baker's yeast (*21*, *22*) and bovine heart muscle (*23*), while P-cytochrome$_{551}$ and cytochrome c show extremely similar absorption spectra (*5*) and normal redox-potentials (*2*). Unlike P-cytochrome oxidase, the cytochrome oxidase containing cytochrome a from bovine heart muscle, solubilized with the aid of cholate can rapidly oxidize cytochrome c's but not P-cytochrome$_{551}$ and P-cytochrome$_{554}$ (*2*).

Therefore, it is most likely that the terminal electron-transferring system is essentially organized by respiratory component-specificities of the respiratory enzymes but not by specificities between the individual components.

From these facts and speculations, the "shortest" electron-transferring system in the oxygen- and nitrate-respirations of *Pseudomonas aeruginosa* is inferred to be as follows:



where the reversible arrows show a possibility that all the respiratory components may functionally be in a state of equilibrium according to their own redox-potentials but not their reductant-oxidant specificities.

SUMMARY

Using whole cells, a sonicated preparation and a cell-free extract of *Pseudomonas aeruginosa*, the functions of the respiratory components present in the cell were studied physiologically.

Besides P-cytochrome oxidase, P-cytochrome$_{551}$, P-cytochrome$_{554}$ and P-blue protein, which have been solubilized and highly purified, it was demonstrated that this bacterium has another cytochrome b_1 type pigment, P-cytochrome$_{(560)}$. This cytochrome as well as the other respiratory components is directly or indirectly functional in oxygen and nitrate respiration. Of these components, there is some evidence that P-cytochrome$_{(560)}$ may be the first cytochrome to accept electrons from succinate. P-cytochrome$_{55}$ may be the first cytochrome to accept electrons from DPNH. P-cytochrome$_{(560)}$ may be most intimately related to nitrate reduction.

Based on these findings, the terminal electron-transfer system of *Pseudomonas aeruginosa* was discussed and a possible scheme of the functional system presented.

The author would like to express his thanks to Prof. K. Okunuki and Dr. T. Horio for their valuable guidance. Thanks are also due to his colleagues Messrs. T. Higashi, M. Nozaki, J. Yamashita and H. Mizushima, for their helpful work and discussions during this study.

REFERENCES

(*1*) Horio, T., *J. Biochem.*, **45**, 195 (1958)
(*2*) Horio, T., *J. Biochem.*, **45**, 267 (1958)
(*3*) Horio, T., Higashi, T., Matsubara, H., Kusai, K., Nakai, M., and Okunuki, K., *Biochim. et Biophys. Acta*, **29**, 297 (1958)
(*4*) Higashi, T., *J. Biochem.*, **45**, 785 (1958)
(*5*) Horio, T., Higashi, T., Nakai, M., Kusai, K., and Okunuki, K., *Nature*, **182**, 1307 (1958)
(*6*) Bach, S. J., Dixon, M., and Zerfas, L. G., *Biochem. J.*, **40**, 229 (1946)
(*7*) Appleby, C. A., and Morton, R. K., *Nature*, **173**, 749 (1954)
(*8*) Yamashita, J., Higashi, T., Yamanaka, T., Nozaki, M., Mizushima, H., Matsubara, H., Horio, T., and Okunuki, K., *Nature*, **179**, 959 (1957)
(*9*) Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.*, **45**, 291 (1958)
(*10*) Yamashita, J., Horio, T., and Okunuki, K., *J. Biochem.*, **45**, 707 (1958)
(*11*) Keilin, D., and Hartree, E. F., *Biochem. J.*, **44**, 205 (1949)
(*12*) Slater, E. C., *3rd Intern. Congr. Biochem., Reports*, p. 89 (1955)
(*13*) Green, D. E., *J. Japan. Biochem. Soc.*, **29**, 65 (1957)
(*14*) Chance, B., *Proc. Intern. Symposium on Enzyme Chem.*, Maruzen, Tokyo, p. 295 (1958)
(*15*) Okunuki, K., Hagihara, B., Sekuzu, I., and Horio, T., *Proc. Intern. Symposium on Enzyme Chem.*, Maruzen, Tokyo, p. 264 (1958)

(*16*) Verhoeven, W., and Takeda, Y., "A Symposium on Inorganic Nitrogen Metabolism", Johns Hopkins Univ. Press, Baltimore, p. 159 (1956)
(*17*) Taniguchi, S., Sato, R., and Egami, F., "A Symposium on Inorganic Nitrogen Metabolism", Johns Hopkins Univ. Press, Baltimore, P. 87 (1956)
(*18*) Treadwell, F., "Analytical Chemistry", 8th Eng. Ed., John Willey and Sons, Inc., **2**, p. 317 (1956)
(*19*) Calvin, M., and Shibata, K., *Biochim. et Biophys. Acta*, **17**, 1 (1955)
(*20*) Pappenheimer, A. M., Jr., and Hendee, E. D., *J. Biol. Chem.*, **180**, 597 (1949)
(*21*) Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.*, **44**, 453 (1957)
(*22*) Nozaki, M., Mizushima, H., Horio, T., and Okunuki, K., *J. Biochem.*, **45**, 815 (1958)
(*23*) Yamanaka, T., Mizushima, H., Nozaki, M., Horio, T., and Okunuki, K., *J. Biochem.*, **46**, 121 (1959)
(*24*) Mizushima, H., Nozaki, M., Horio, T., and Okunuki, K., *J. Biochem.*, **45**, 845 (1958)
(*25*) Boeri, E., Cutolo, E., Luzzati, M., and Tosi, L., *Arch. Biochem. Biophys.*, **56**, 478 (1955)
(*26*) Strittmatter, P., and Velick, S. F., *J. Biol. Chem.*, **228**, 785 (1957)
(*27*) Raw, I., Molinari, R., doAmaral, D. F., and Mahler, H. R., *J. Biol. Chem.*, **233**, 225 (1958)
(*28*) Mahler, H. R., Raw, I., Molinari, R., and doAmaral, D. F., *J. Biol. Chem.*, **233**, 230 (1958)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

GLUTAMIC ACID FORMATION FROM GLUCOSE BY BACTERIA

I. ENZYMES OF THE EMBDEN-MEYERHOF-PARNAS PATHWAY, THE KREBS CYCLE, AND THE GLYOXYLATE BYPASS IN CELL EXTRACTS OF BREVIBACTERIUM FLAVUM No. 2247.

By ISAMU SHIIO, SHIN-ICHIRO ÔTSUKA and TOSHINAO TSUNODA

(From the Central Research Laboratory of Ajinomoto Co., Inc., Kawasaki)

(Received for publication, March 5, 1959)

In our laboratory, a strain of *Brevibacterium flavum*, No. 2247, which grew on glucose as the sole source of carbon and accumulated a large amount of L-glutamate in its growing culture medium, has been isolated from a rotten onion. The authors' preliminary studies* on the pathway of glutamate formation showed that by the cell suspension of this bacterium a large amount of α-ketoglutaric acid or glutamic acid (in the presence of ammonium salts) was aerobically formed not only from glucose but also from acetate and that these products were not further degraded. It was also observed that pyruvic acid was aerobically produced from glucose in the presence of arsenite. Since the Krebs cycle seems to be the chief pathway in the various organisms for the formation of α-ketoglutaric acid from pyruvic acid and the glyoxylate cycle, recently proposed by Kornberg and Krebs (*1*), accounts for the net synthesis of α-ketoglutaric acid from acetate when this is the sole source of carbon, it is of interest to demonstrate in the cell free extracts the presence of various enzymes of these pathways as well as of the Embden-Meyerhof-Parnas pathway, which may operate in the formation of pyruvate from glucose.

The present paper reports on the demonstration of these enzymes in the cell free extracts of this strain of *Brevibacterium flavum*, No. 2247.

METHODS

Cell Free Extract.—Brevibacterium flavum No. 2247 was grown on a shaker at 30° in 500 ml. flasks containing 50 ml. of medium with following composition: glucose 36 g., urea 10 g., KH_2PO_4 1 g., $MgSO_4 \cdot 7\,H_2O$ 0.4 g., $FeSO_4 \cdot 7H_2O$ 10 mg., $MnSO_4 \cdot 4H_2O$ 8.13 mg., thiamine hydrochloride 100 μg., biotin 2 μg., Ajinomoto's "Mieki" (aquous solution of amino acid mixture prepared from HCl-hydrolysate of soybean meal, 22 g. of nitrogen per liter) 1 ml. per liter, pH 7.0. After 24 hours the cells were harvested by centrifugation,

* unpublished data.

washed twice with 0.2 per cent KCl, suspended in 0.01 *M* Tris* or phosphate buffer, pH 7.5, and disintegrated by treatment with a sonic oscillator (" Toyorika Type 50-2 ", 9 kc, 80 w.) for 30 minutes under cooling. The cell debris was removed by centrifugation at $18,000 \times g$ for 30 minutes at low temperature and the clear supernatant fluid (cell free extract), which contained usually 0.5 to 0.7 mg. of nitrogen per ml. was used for the demonstration of various enzyme activities.

Cell-free extract was also prepared from dried cells. Washed cells were dried up over P_2O_5 *in vacuo*, suspended in 0.01 *M* Tris or phosphate buffer pH 7.5 for one or two hours, and centrifuged at $18,000 \times g$ for 30 minutes.

α-ketoglutaric acid, trisodium isocitrate, DPN, TPN, and barium salts of G-6-P, F-6-P and FDP were obtained from Nutritional Biochemicals Corporation. These sugar phosphates were converted to potassium salts after some purification procedures (*2*). Oxalacetic acid, *cis*-aconitic acid, glyoxylic acid dihydrate, and ATP were obtained from L. Light and Co., Ltd., Fluka and Co., Dr. T. Schuchardt GMBH and Co., and Zellstofffabrik Waldhof, respectively.

Method for the Demonstration of Various Enzyme Activities

G-6-P Dehydrogenase—The method used was based on the spectrophotometric measurement of the reduction of TPN at 340 mμ (*3*).

Hexokinase—A spectrophotometric method based on the conversion of glucose to G-6-P which was dehydrogenated by TPN in the presence of G-6-P dehydrogenase was used.

Aldolase and GAP Dehydrogenase—The rate of reduction of DPN (or TPN) resulting from GAP oxidation that was formed FDP by the aldolase action was measured, using a spectrophotometer at 340 mμ as described by Warburg and Christian (*4*).

Phos hohexokinase—The formation of FDP from F-6-P was measured by a spectrophotometric method involving the conversion of FDP to GAP and the reduction of DPN resulting from GAP oxidation in the presence of aldolase, GAP dehydrogenase and arsenate.

Phosphohexose Isomerase—The formation of F-6-P from G-6-P was measured by a spectrophotometric method involving the conversion of F-6-P to FDP and of FDP to GAP and the reduction of DPN resulting from GAP oxidation in the presence of phosphohexokinase, aldolase, GAP dehydrogenase, ATP, magnesium ions, and arsenate. A colorimetric method based on the conversion of G-6-P to F-6-P, which gives about 65 per cent of the color of free fructose in the modified resorcinol method of Roe (*5*, *6*) was also used.

Isocitric Dehydrogenase—The method used was essentially that of Ochoa (*7*) and depended on the spectrophotometric determination of TPN reduction in the presence of magnesium or manganese ions.

Aconitase—The method used was based on the light absorption, at 240 mμ. of *cis*-aconitic acid formed from citrate or DL-isocitrate (*8*). Another method used was based on measurement of the reduction of TPN by isocitric acid formed from *cis*-aconitate or citrate *via cis*-aconitate in the presence of isocitric dehydrogenase and manganese ions.

Succinic Dehydrogenase—A manometric method based on the measurement of oxygen

* The abbreviations used throughout this paper include Tris, tris(hydroxymethyl)-aminomethane ; TPN, triphosphopyridine nucleotide ; DPN, diphosphopyridine nucleotide ; DiNPH, 2,4-dinitrophenylhydrazine or 2,4-dinitrophenylhydrazone ; TCA, trichloroacetic acid ; GSH, glutathione ; G-6-P, glucose-6-phosphate ; F-6-P, fructose-6-phosphate ; GAP, glyceraldehyde-3-phosphate ; FDP, fructose-1,6-diphosphate ; αKG, α-ketoglutaric acid ; ATP, adenosine triphosphate ; ΔE_{340}, increment of optical density at 340 mμ ; and EMP pathway, Embden-Meyerhof-Parnas pathway.

consumption during succinate oxidation in the presence of cyanide and methylene blue was used (*9*), and a spectrophotometric method measuring the reduction rate of $K_3Fe(CN)_6$ in the presence of sufficient KCN to inhibit cytochrome oxidase was also used (*10*).

Malic Dehydrogenase or "Malic" Enzyme—The rate of reduction of TPN resulting from L-malic acid oxidation was followed in a spectrophotometer at 340 mμ.

Fumarase—A spectrophotometric method based on the high ultraviolet absorption of fumarate decreased or formed from L-malate at 295 mμ and 240 mμ, respectively (*11*). Another method used was based on measurement of the reduction of TPN by malate formed from fumarate in the presence of malic dehydrogenase (or malic enzyme) and manganese ions.

Oxalacetic Carboxylase—The CO_2 evolved when oxalacetate is decarboxylated by the enzyme was measured manometrically.

Isocitritase—Glyoxylic acid formed was determined in mixtures of glyoxyric acid and αKG as its DiNPH by the modified colorimetric method of Friedemann and Haugen (*12*), taking into account the ratio of absorption at 450 mμ and 550 mμ. Glyoxylic acid and αKG in the standard solutions were estimated iodometrically by the method of Crift and Cook (*13*).

Detailed conditions of the enzyme reactions above described will be given in Table I, II, and Fig. 1, respectively.

The formation of keto acids and non volatile organic acids in the various reactions was demonstrated paperchromatographically by using *n*-butanol—ethanol—0.5 *N* NH_4OH (7 : 1 : 2); *n*-butanol—formic acid—water (4 : 1.5 : 1) and phenol—water (10 : 2 *v/v*) as the solvent systems, respectively, after the treatment of the reaction mixtures as described in our previous reports (*14*, *15*).

The protein-N content of cell extracts was determined by the micro-Kjeldahl method or the method of Lowry *et al.*, using the Folin-Ciocalteu reagent (*16*).

RESULTS

Glucose-6-phosphate Dehydrogenase—Spectrophotometric studies of the reduction of TPN by G-6-P with cell free extracts demonstrated the existence of G-6-P dehydrogenase (Table I). This dedydrogenase was TPN-specific and required magnesium ions for maximum activity.

Enzymes of the Embden-Meyerhof-Pathway—Having established the presence of a TPN-specific G-P-P dehydrogenase in the cell extracts, reduction of TPN with glucose in the presence of ATP indicates the presence of hexokinase activity in the cell extracts (Table I). In the case of this extract of the dried cells, reduction of TPN occurred considerably also in absence of ATP. When FDP was incubated with the cell extracts under the conditions essential for the demonstration of GAP dehydrogenase, *i.e.*, in the presence of glutathione and arsenate, reduction of DPN could be demonstrated spectrophotometrically. Since G-6-P dehydrogenase has been shown to be TPN-specific, DPN reduction under these conditions indicates the presence of the enzymes, aldolase and GAP dehydrogenase. This enzyme system was DPN-specific and required glutathione and arsenate for maximum activity (Table I). DPN reduction under these conditions occurred also on incubation of F-6-P or G-6-P with the cell extracts in the presence of ATP and magnesium ions, indicating the presence of phosphohexokinase and phosphohexose

TABLE I

Demonstration of the Enzymes of the Embden-Meyerhof-Parnas Pathway and Glucose-6-phosphate Dehydrogenase in the Cell Free Extracts of Brevibacterium flavum No. 2247

Enzyme related	Substrate	Reaction mixture and condition	Reaction rate
G-6-P dehydrogenase	G-6-P	(R-I), TPN, 23°	2.3×10^{-3} ΔE_{340} per min.
	,,	(R-I), DPN, ,,	0.0 ,,
	,,	(R-I)-G-6-P, TPN, ,,	0.1 ,,
	,,	(R-I)-G-6-P, DPN, ,,	0.0 ,,
G-6-P dehydrogenase	G-6-P	(R-I'), 20°	3.6×10^{-2} ΔE_{340} per min.
	,,	(R-I')-$MgCl_2$, ,,	0.18 ,,
	,,	(R-I')-G-6-P, ,,	0.06 ,,
	,,	(R-I')-cell ext., ,,	0.00 ,,
Hexokinase and G-6-P dehydrogenase	Glucose	(R-II), 20°	9.4×10^{-3} ΔE_{340} per min.
	,,	(R-II)-$MgCl_2$, ,,	0.0 ,,
	,,	(R-II)-glucose, ,,	0.0 ,,
Aldolase and GAP dehydrogenase	FDP	(R-III), DPN, 23°	7.0×10^{-2} ΔE_{340} per min.
	,,	(R-III), TPN, ,,	0.2 ,,
	,,	(R-III)-FDP, DPN, ,,	0.0 ,,
	,,	(R-III)-FDP, TPN, ,,	0.0 ,,
Aldolase and GAP dehydrogenase	FDP	(R-III'), 20°	2.0×10^{-2} ΔE_{340} per min.
	,,	(R-III')-glycine, ,,	2.0 ,,
	,,	(R-III')-GSH, ,,	0.45 ,,
	,,	(R-III')-arsenate, ,,	0.0 ,,
	,,	(R-III')-FDP, ,,	0.0 ,,
	,,	(R-III')-cell ext., ,,	0.0 ,,
Phosphohexokinase, aldolase and GAP dehydrogenase	F-6-P	(R-IV), 20°	1.1×10^{-2} ΔE_{340} per min.
	,,	(R-IV)-ATP, ,,	0.07 ,,
	,,	(R-IV)-$MgCl_2$, ,,	0.00 ,,
	,,	(R-IV)-arsenate, ,,	0.73 ,,
	,,	(R-IV)-F-6-P, ,,	0.05 ,,
Phosphohexose isomerase, phosphohexokinase, aldolase, and GAP dehydrogenase	G-6-P	(R-V), 20°	8.5×10^{-3} ΔE_{340} per min.
	,,	(R-V)-ATP, ,,	0.1 ,,
	,,	(R-V)-$MgCl_2$, ,,	0.0 ,,
	,,	(R-V)-arsenate, ,,	5.4 ,,
	,,	(R-V)-G-6-P, ,,	0.1 ,,
Phosphohexose isomerase	G-6-P	(R-V'), 37°	5.4×10^{-2} μM F-6-P per min.
	,,	(R-V')-G-6-P, ,,	0.0 ,, per ml.

Reaction mixtures contained the following reactants in micromoles per ml.

(R-I) : G-6-P 10; $MgCl_2$ 10; Tris buffer, pH 7.5, 50; TPN or DPN 0.25; and cell extract(I) 0.15 mg. N.

(R-I') : G-6-P 10; $MgCl_2$ 10; Tris buffer, pH 7.5, 50; TPN 0.125; and cell extract (II) (dialysed) 0.15 mg. N.

(R-II) : glucose 10; $MgCl_2$ 10; ATP 5; Tris buffer, pH 7.5, 50; TPN 0.125; and cell extract (III) (dried cell extract) 0.50 mg. N.

(R-III) : FDP 12; sodium arsenate 10; glycine 25; GSH 12.5; Tris buffer, pH 7.5, 20; TPN or DPN 0.25; and cell extract (I) 0.15 mg. N.

(R-III') : FDP 10; sodium arsenate 10; glycine 25; GSH 12.5; Tris buffer, pH 7.5, 20; DPN 0.125; and cell extract(II) (dialysed) 0.15 mg. N.

(R-IV) : G-6-P 10; ATP 5; $MgCl_2$ 10; sodium arsenate 10; glycine 25; GSH 12.5; Tris buffer, pH 7.5, 15; DPN 0.125; and cell extract (IV) (dialysed) 0.14 mg. N.

(R-V) : This was the same as above (R-IV) except for G-6-P as a substrate.

(R-V') : G-6-P 20; Tris buffer, pH 7.5, 50; cell extract (VIII) 0.015 mg. N.

isomerase. The presence of the latter enzyme was also demonstrated by the method of Roe (*5*), by which enzymatically formed ketose phosphate was estimated (Table I).

Enzymes of the Krebs Cycle—Spectrophotometric studies of the formation of aconitate from citrate and isocitrate and of the hydration and the formation from L-malate of fumarate demonstrated the existence of aconitase and fumarase, respectively (Table II). The reversible conversion between fumarate and malate was also demonstrated by the paperchromatography of non volatile acids in the forward and reverse reaction mixtures. TPN reduction occurred on incubation of isocitrate or L-malate with the cell extracts in the presence of manganese ions, indicating the presence of isocitric dehydrogenase and malic dehydrogenase (or malic enzyme), respectively. Both dehydrogenases were TPN-specific and required manganese ions for full activity (Table II). Under these conditions TPN reduction occurred also on incubation of citrate, *cis*-aconitate, or fumarate with the cell extracts, indicating the presence of aconitase and fumarase (Table II).

The presence of succinic dehydrogenase in the cell free extract was demonstrated manometrically and spectrophotometrically in the presence of sufficient KCN to inhibit cytochrome oxidase (Table II). These tests for this enzyme were also carried out on the cloudy supernatant fluid obtained by centrifuging for 30 minutes at $1{,}500 \times g$ after the sonic treatment and the same cell suspension as that used for the preparation of the cell free extract above described. The relative reaction rates for the clear cell-free extract, the cloudy supernatant, and the intact cells, were:

100 : 161 : 132 (manometric method) and
100 : 113 : — (spectrophotometric method).

Oxalacetic Carboxylase—As shown in Table II, the cell extracts indicated high activity of oxalacetic carboxylase.

Isocitritase—The presence of isocitritase was demonstrated by determining glyoxylic acid formed during the incubation of citrate with the cell extract as shown in Fig. 1. This enzyme required magnesium ions but no glutathione for full activity. Glyoxylic acid was identified as a product of the isocitritase reaction by the chromatographic behaviour, alkali instability, and the characteristic ultraviolet spectrum of its DiNPH (Fig. 2). The solvent systems used in this paperchromatography (0.05 *N* NaOH; *n*-butanol saturated with 3 per cent ammonia; *n*-butanol—ethanol—0.5 *N* NH_4OH (7 −1 −2); phenol—water (10 : 2)) could distinguish glyoxylic acid from αKG, pyruvate, oxalacetate, β-formylpropionate, and α-ketoisovalerate, and chromatography of the keto acid DiNPH from reaction mixtures showed also no perceptible trace of any keto acid other than glyoxylic and α-ketoglutaric acids, the latter of which was also identified by the ultraviolet spectrum of its DiNPH. The formation of glyoxylate and αKG was also detected in the case of isocitrate as a substrate.

Succinic acid was identified as another product of the isocitritase reaction by chromatography. After 5 hours of incubation at 37°, the reactian mixture, which contained 30 μM of sodium citrate, 3.3 μM of $MgCl_2$, 33 μM of Tris buffer,

TABLE II

Demonstration of the Enzymes of the Krebs Cycle and Oxalacetic Carboxylase in the Cell Free Extracts of Brevibacterium flavum No. 2247

Enzyme related	Substrate	Reaction mixture and condition	Reaction rate
Aconitase	Citrate	(R-VI), 20°	2.0×10^{-2} ΔE_{240} per min.
	DL-Isocitrate	(R-VI), "	5.6×10^{-2} ΔE_{240} per min.
		(R-VI)-substrate, "	0.00 "
Isocitric dehydrogenase	Isocitrate	(R-VII), TPN, 23°	2.2×10^{-2} ΔE_{340} per min.
	"	(R-VII'), DPN, "	0.15 "
	"	(R-VII)-substrate, "	0.0 "
	"	(R-VII')-substrate, "	0.0 "
Isocitric dehydrogenase	Isocitrate	(R-VII''), 20°	1.7×10^{-2} ΔE_{340} per min.
	"	(R-VII'')-$MgCl_2$, "	1.7 "
	"	(R-VII'')-$MnCl_2$, "	0.9 "
	"	(R-VII'')-($MnCl_2$+ $MgCl_2$), 20°	0.07 "
	"	(R-VII'')-substrate, "	0.00 "
	"	(R-VII'')-cell ext., "	0.00 "
Aconitase and isocitric dehydrogenase	Citrate	(R-VIII), 23°	1.7×10^{-1} ΔE_{340} per min.
	cis-Aconitate	(R-VIII), "	0.5 "
		R-VIII)-substrate, "	0.0 "
Succinic dehydrogenase	Succinate	(R-IX), 37°	1.00 μl. O_2 uptake per min.
	"	(R-IX)-substrate, "	0.00 "
Succinic dehydrogenase	Succinate	(R-IX'), 20°	7.0×10^{-3} ($-\Delta E_{400}$) per min.
	"	(R-IX')-substrate, "	0.0 "
Fumarase	Fumarate	(R-X), 20°	5.3×10^{-2} ($-\Delta E_{295}$) per min.
	L-Malate	(R-X), "	1.2×10^{-2} ΔE_{240} per min.
		(R-X)-substrate, "	0.0 "
Malic dehydrogenase (or malic enzyme)	L-Malate	(R-XI), TPN, 23°	2.9×10^{-2} ΔE_{340} per min.
	"	(R-XI), DPN, "	0.02 "
	"	(R-XI)-malate, TPN, "	0.01 "
	"	(R-XI)-malate, DPN, "	0.00 "
Malic dehydrogenase (or malic enzyme)	L-Malate	(R-XI'), 20°	2.6×10^{-3} ΔE_{340} per min.
	"	(R-XI')-$MnCl_2$, "	0.2 "
	"	(R-XI')-malate, "	0.0 "
	"	(R-XI')-cell ext., "	0.1 "
Fumarase and malic dehydrogenase (or malic enzyme)	Fumarate	(R-XI''), 23°	2.3×10^{-3} ΔE_{340} per min.
	"	(R-XI'')-$MnCl_2$, "	0.7 "
	"	(R-XI'')-fumarate, "	0.1 "
Oxalacetic carboxylase	Oxalacetate	(R-XII), 37°	13* μl. CO_2 per min.

Reaction mixtures contained the following reactants in micromoles per ml.

(R-VI) : substrate 10; Tris buffer, pH 7.5, 25; and cell extract (VIII) 0.015 mg. N.

(R-VII) : sodium iocitrate 20; $MgCl_2$ 5; $MnCl_2$ 0.5; Tris buffer, pH 7.5, 50; TPN 0.125; and cell extract (I) 0.0015 mg. N.

(R-VII') : sodium isocitrate 20; $MgCl_2$ 5; $MnCl_2$ 0.5; Tris buffer, pH 7.5, 50; DPM 0.25; and cell extract (I) 0.15 mg. N.

(R-II'') : sodium isocitrate 10; $MgCl_2$ 5; $MnCl_2$ 0.5; Tris buffer, pH 7.5, 50; TPN 0.0125; and cell extract (II) (dialysed) 0.003 mg. N.

(R-VIII) : substrate 10; $MnCl_2$ 0.5; Tris buffer, pH 7.5, 50; TPN 0.125; and cell extract (VI) (dialysed) 0.15 mg. N.

(R-IX) : sodium succinate 40; KCN 10; phosphate buffer, pH 7.5, 80; methylene blue 1; and cell extract (V) 0.11 mg. N., total volume was 2.5 ml. in a Warburg flask under air.

(R-IX') : sodium succinate 10; KCN 10; phosphate buffer, pH 7.5, 100; $K_3Fe(CN)_6$ 0.4; and cell extract (V) 0.14 mg. N.

(R-X) : sodium fumarate 10 or sodium L-malate 50; phosphate buffer, pH 7.5, 50; and cell extract (V) 0.14 mg. N.

(R-XI) : sodium L-malate 10; $MnCl_2$ 0.5; Tris buffer, pH 7.5, 50; TPN or DPN 0.25; and cell extract (I) 0.15 mg. N.

(R-XI') : sodium L-malate 10; $MnCl_2$ 0.5; Tris buffer, 7.5, 50; TPN 0.125; and cell extract (II) (dialysed) 0.15 mg. N.

(R-XI''): sodium fumarate 10; $MnCl_2$ 0.5; Tris buffer, pH 7.5, 50; TPN 0.125; and cell extract (VI) (dialysed) 0.15 mg. N.

(R-XII) : sodium oxalacetate 40; phthalate buffer, pH 4.8, 80; and cell extract (VII) 0.25 mg. N., total volume was 2.5 ml. in a Warburg flask under air.

* A value obtained in absence of substrate has been substracted to give above value.

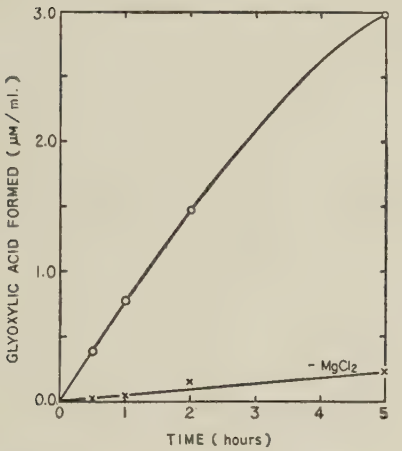


FIG. 1. Formation of glyoxylic acid from citrate by cell free extract of *Brevibacterium flavum* No. 2247.

Complete system of reaction contained 30 μM of sodium citrate, 5 μM of $MgCl_2$, and 0.29 mg. nitrogen of cell extract in a final volume of 1 ml. of 0.05 *M* Tris buffer, pH 7.5. After the incubation at 37°, the reaction was stopped by addition of 2 ml. of 10 per cent TCA and glyoxylate formed was determined by the modified method of Friedemann and Haugen.

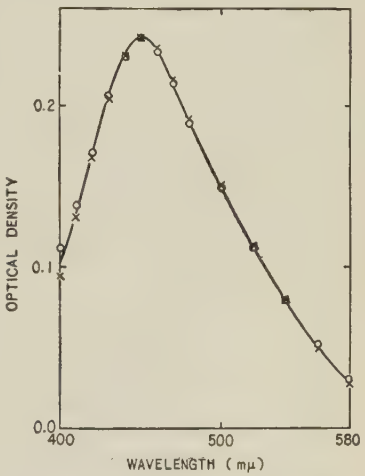


FIG. 2. Ultraviolet absorption spectra of 2,4-dinitrophenyl hydrazones of glyoxylic acid (—×—) and isocitritase reaction product (—○—) in 1 *N* sodium hydroxide.

pH 7.5, and 0.40 mg. nitrogen of the cell extract per ml. was acidified, dried up, and extracted with ether. The ether was evaporated and the residue was chromatographed on Toyo No. 51 filter paper with *n*-butanol—formic acid—water (4 : 1.5 : 1) and phenol—water (10 : 2). After removal of volatile acids by air drying, the fixed acids revealed by 0.1 per cent bromophenol blue spray were succinic and citric acid.

DISCUSSIONS

Various glycolytic enzymes which serve in the EMP pathway have been

demonstrated in the cell free extracts of *Brevibacterium flavum* No. 2247, and the presence of phosphohexokinase and aldolase specific for FDP, which are unique to the EMP pathway (*17*), may be cited as the evidence for this pathway. All other enzymes of the EMP pathway are also common to other known pathways of both aerobic and anaerobic carbohydrate metabolism. On the other hand, the presence of TPN-linked G-6-P dehydrogenase suggests the possible operation of hexose monophosphate pathways in this bacterium. All the enzymes of the Krebs cycle except for condensing enzyme and αKG dehydrogenase which were not examined in our present studies, have been demonstrated in the cell free extracts. This fact suggests the operation of the Krebs cycle in the cells. The presence of aconitase and isocitric dehydrogenase seems to provide a pathway for the formation of αKG from pyruvate.

An alternative pathway may be as follows: Fission of glucose to triose phosphates, oxidation to pyruvate, the fixation of carbon dioxide to form oxalacetate, the condensation of this with acetyl coenzyme A derived from another molecule of pyruvate, isomerization of citrate formed to isocitrate, which is then oxidatively decarboxylated. This pathway cannot, however, account for the synthesis observed of αKG from acetate. The occurrence of isocitritase in the cell extract suggests that the accumulation of αKG involves the modified Krebs cycle with "glyoxylate bypass" (*1*), the intermediate stages being:

$$\text{isocitrate} \longrightarrow \text{glyoxylate} + \text{succinate}$$
$$\text{glyoxylate} + \text{acetate} \longrightarrow \text{malate}$$
$$\text{malate} + 1/2\ O_2 \longrightarrow \text{oxalacetate}$$
$$\text{succinate} + O_2 \longrightarrow \text{oxalacetate}$$
$$2\ \text{acetate} + 2\ \text{oxalacetate} \rightarrow 2\ \text{isocitrate}$$
$$\text{isocitrate} + 1/2\ O_2 \longrightarrow \alpha\text{-ketoglutarate} + CO_2$$
$$\text{Sum}: 3\ \text{acetate} + 2\ O_2 \longrightarrow \alpha\text{-ketoglutarate} + CO_2$$

Succinic dehydrogenase activity of the cloudy supernatant fluid obtained by centrifuging at $1{,}500 \times g$ after the sonic treatment was rather higher than that of the intact cell suspension. This fact indicates that the enzyme activity of the supernatant fluid can not be attributed to a trace of the intact cell that may exist. The difference of the enzyme activity between this supernatant fluid and the usual cell extract obtained by centrifuging at $18{,}000 \times g$ was larger in the manometric method than in the ferricyanide method, and may be attributed to the portion precipitated during the centrifugation process at $18{,}000 \times g$ after at $1{,}500 \times g$. Since in the oxygen uptake cytochrome b in addition to succinic dehydrogenase and methylene blue seems to be involved and soluble purified succinic dehydrogenase from beef heart mitochondria has been known to react directly with ferricyanide but not with methylene blue (*9*, *18*), the precipitates above described seem to contain cytochromes rather than the dehydrogenase itself.

SUMMARY

1. An evidence was obtained for the presence of hexokinase, phosphohexose isomerase, phosphohexokinase, aldolase, DPN-linked glyceraldehyde-3-

phosphate dehydrogenase, TPN-linked glucose-6-phosphate dehydrogenase, aconitase, TPN-linked isocitric dehydrogenase, succinic dehydrogenase, fumaras, TPN-linked malic dehydrogenase (or malic enzyme), and oxaloacetic carboxylase in the cell free extracts of a strain of *Brevibacterium flavum*, No. 2247.

2. The presence of isocitritase, one of the enzymes of the "glyoxylate bypass", was also demonstrated and glyoxylic acid and succinic acid were identified as products of the isocitritase reaction by chromatography and by ultraviolet spectrum.

The authors are indebted to Prof. S. Akabori of the Osaka University and Dr. H. Oeda, Dr. T. Ogawa, and Mr. N. Motozaki of our company for the helpful criticism and encouragement during the course of this work.

REFERENCES

(*1*) Kornberg, H. L., and Krebs, H. A., *Nature*, **179**, 989 (1957)
(*2*) Neuberg, C., Lustig, H., and Rothenberg, M. A., *Arch. Biochem.*, **3**, 33 (1943)
(*3*) Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936)
(*4*) Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939)
(*5*) Roe, H. J., *J. Biol. Chem.* **107**, 15 (1934)
(*6*) Slein, M. W., Methods in Enzymology, Academic Press Inc., Publishers, New York, Vol. I, p. 301 (1955)
(*7*) Ochoa, S., *J. Biol. Chem.*, **174**, 133 (1948)
(*8*) Racker, E., *Biochim. et. Biophys. Acta*, **4**, 211 (1950)
(*9*) Slater, E. C., *Biochem. J.*, **45**, 1 (1949)
(*10*) Slater, E. C., and Bonner, W. D., *Biochem. J.*, **52**, 185 (1952)
(*11*) Racker, E. C., *Biochim. et Biophys. Acta*, **4**, 20 (1950)
(*12*) Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943)
(*13*) Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932)
(*14*) Tsunoda, T., and Shiio, I., *J. Biochem.*, **46**, 1011 (1959)
(*15*) Tsunoda, T., and Shiio, I., *J. Biochem.*, **46**, 1227 (1959)
(*16*) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951)
(*17*) Gunsalus, I. C., Horecker, B. L., and Wood, W. A., *Bacteriol. Rev.*, **19**, 79 (1955)
(*18*) Singer, T. P., and Lara, F. J. S., *Proc. Intern. Symposium on Enzyme Chem.*, p. 330 (1957)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

BILE ACIDS AND STEROIDS

XI. SYNTHESIS OF 3α,6α,12α-TRIHYDROXYCHOLANIC ACID AND ITS OXIDATION PRODUCTS*

By KEN'ICHI TAKEDA and KIKUO IGARASHI

(From the Research Laboratory, Shionogi & Co., Ltd., Imafuku, Amagasaki, Hyohgo-ken)

(Receievd for publication, March 6, 1959)

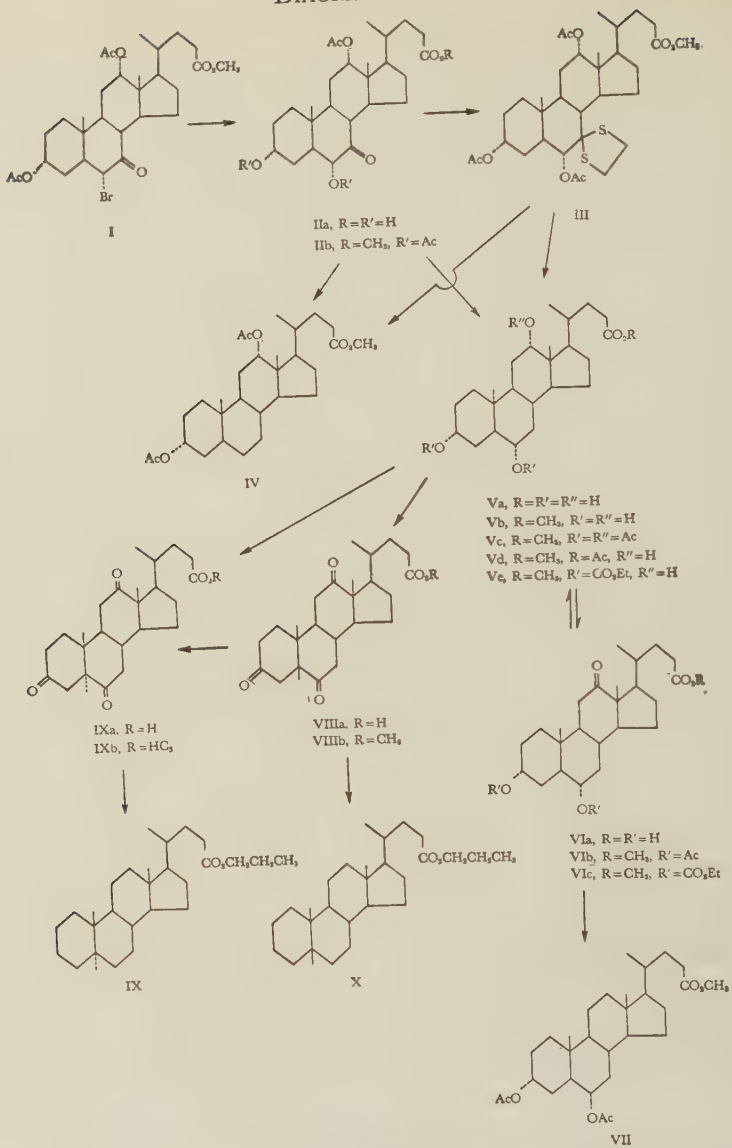
In the previous papers, (*2*), (*3*) we reported that methyl 3α,12α-diacetoxy-6α-bromo-7-oxocholanate gave 3α,6ζ,12α-trihydroxy-7-oxocholanic acid by the saponification under nitrogen at room temperature and that the conformation of the achieved C-6 hydroxyl group was assumed to be equatorial (α).

On the other hand, in 1949, a new trihydroxycholanic acid was obtained by the permanganate oxidation of the tetrahydroxy-norsterocholanic acid, which was isolated from the bile of "gigi"-fish or "fugu"-fish, by K. Ohota (*4*) and H. Isaka, (*5*) and they assumed this new cholanic acid as 3α,6α,12α-trihydroxycholanic acid from the results of its degradation products, and the name "iso-cholic acid" was given to this compound by Ohta (*4*), one of the abovementioned authors.

Now we prepared 3α,6α,12α-trihydroxycholanic acid from 3α,6ζ,12α-trihydroxy-7-oxocholanic acid and also clarified the C-6 hydroxyl group, which was introduced by the saponification of the 6α-bromo compound, to have an equatorial conformation (α) as expected earlier. Huang-Minlon reduction of methyl 3α,6ζ,12α-triacetoxy-7-oxocholanate (IIb) afforded methyl 3α,6ζ,12α-triacetoxycholanate (Vc) with a small amount of methyl desoxycholate diacetate (IV) when the reduction mixture was esterified, acetylated and purified by chromatography on alumina. An oily product and a small amount of methyl 3α,12α-diacetoxy cholanate were obtained from the first benzene-petroleum ether eluate. The anticipated methyl 3α,6ζ,12α-triacetoxycholanate (Vc), m.p. 150-151°, was obtained from the next benzene-petroleum ether and benzene eluates in a 50 per cent yield. Another oily substance was also obtained from the last eluate but was not investigated further. The thioketal derivative of IIb also gave the same compound (Vc) in a 20 per cent yield by the action of Raney nickel in boiling methanol, but, in this case the principal reduction product was the methyl desoxycholate diacetate (IV) (80 per cent yield). Saponification of this methyl ester triacetate (Vc) gave an oily acid (Va), which resisted all attempts for crystallization.

* See (*1*)

DIAGRAMM 1



In order to confirm the configuration of the C-6 hydroxyl group, the following experiments were attempted. Partial acetylation of the methyl ester of trihydroxycholanic acid (Vb) with acetanhydride-pyridine in benzene according to the procedure employed to prepare methyl cholate 3,7-diacetate (*6*), gave methyl triacetoxycholanate (Vc) as a main product and the anticipated 3α,6ζ-diacetate (Vd), m.p. 154–155° was obtained only in a 30 per cent yield. The poor yield of this diacetate is in marked contrast when compared

with that in the case of the partial acetylation of methyl cholate (*6*).

Methyl 3α,6ζ-diacetoxy-12-oxocholanate (VIb), m.p. 182–183°, resulted from the above-mentioned diacetate (Vd) by treatment with potassium chromate in acetic acid. Its free acid (VIa) melted at 255–256°. Huang-Minlon reduction of this oxo ester (VIb) followed by esterification and acetylation afforded a compound of a melting point 99–101°; this substance was shown to be identical with the authentic sample of methyl α-hyodesoxycholate diacetate (VII) by the mixed melting point behavior and by the infrared spectra. From these results the following facts are confirmed: (1) the diacetate (Vd) which was prepared from the methyl ester (Vb) is 3,6-diacetate and (2) the configuration of the C-6 hydroxyl group is α-oriented (equatorial).

As the yield of 3α,6α-diacetate is not satisfactory, an attempt was made to prepare the 3,6-diethoxycarbonyl derivative (Ve). Treatment of methyl trihydroxy-cholanate (Vb) with ethyl chlorocarbonate in a usual manner furnished a diethoxycarbonyl derivative (Ve) as a viscous oil, in good yield. Ve afforded methyl diethoxycarbonyloxocholanate (VIc), m.p. 160–161°, by potassium chromate oxidation. This compound was characterized by the conversions to its free acid and to its methyl ester diacetate, which were identical with 3α,6α-dihydroxy-12-oxocholanic acid (VIa) and its methyl ester diacetate (VIb) respectively. These experiments also indicate that in this reaction cathylation occurs in both 3 and 6 positions giving a further support to the α-configuration of the C-6 hydroxyl group (*7*). In spite of the facts that each conformation of these hydroxyl groups (3α,6α and 12α) is equatorial, equatorial and axial, respectively, while that of cholic acid (3α,7α and 12α) equatorial, axial and axial, respectively, the low yield of the diacetate of our acid (Vd) is incomprehensible.

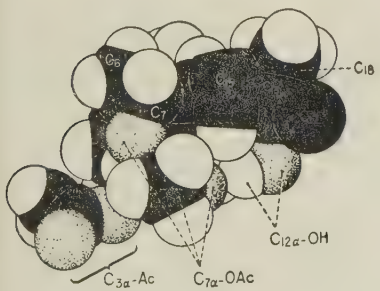


FIG. 1. a. Me 3α,7α-diacetoxy-12α-hydroxycholanate

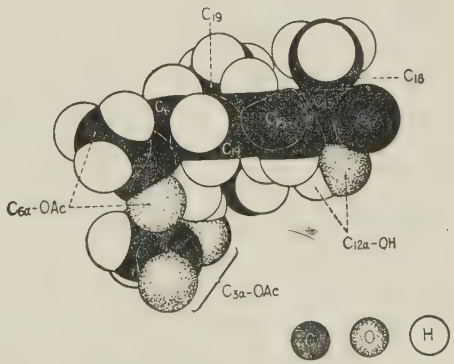


FIG. 1. b. Me 3α,6α-diacetoxy-12α-hydroxycholanate

Our elucidation of these findings is as follows: On the acetylation of cholic acid, the 3α-hydroxyl group (e) and the less hindered 7α-hydroxyl group are acetylated faster than the more hindered 12α-hydroxyl group. At this stage, the 12α-hydroxyl group is strictly hindered by the formed 7α-

acetoxy group, as is evident from models (Fig. 1-a), thus resisting further acetylation. On the other hand, as there is no remarkable interaction between 12α-hydroxyl group and 6α-acetoxy group (e) of 3α,6α,12α-trihydroxycholanic acid (Va), the full acetylation of this acid proceeds more easily than cholic acid (Fig. 1-b).

The reduction of this 12-oxo compound to its corresponding alcohol was then examined. But all attempts, for instance, catalytic reductions using Raney nickel or platinum oxide, sodium borohydride, sodium in propanol and lithium in alcohol and liquid ammonia, failed to give the 12β epimer.

When 3α,6α,12α-trihydroxycholanic acid was oxidized with chromium trioxide in glacial acetic acid, a tri-oxo acid, m.p. 221–223°, was obtained. The structure was deduced to be 3,6,12-trioxocholanic acid (VIIIa) from the results of the analytical values and the infrared absorption spectrum. Its methyl ester (VIIIb) melted at 182–183°. Treatment of this trioxocholanic acid with an aqueous sodium carbonate solution on a water bath for an hour gave 3,6,12-trioxoallocholanic acid (IXa), m.p. 239–241°. This acid was recovered also from the mother liquor of the above-mentioned oxidation. This acid gave a methyl ester (IXb), m.p. 221–222°, treated with diazomethane.

Table I

By authors	m.p.	α_D	By Isaka	m.p.
3α,6α,12α-Trihydroxy cholanic acid	Syrup	+17.8	Iso-cholic acid	207–208
3α,6α-Diacetoxy-12-hydroxycholanic acid	Syrup		Diacetoxy-(3,6?)-monohydroxy cholanic acid	240
Me 3α,6α-diacetoxy-12α-hydroxy cholanate	145–155	+20.3		
Me 3α,6α,12α-Triacetoxy cholanate	150-151	+58.6		
3α,6α-Dihydroxy-12-oxocholanic acid	255–256	+62.4	Dihydroxy-monooxo-cholanic acid	188–190
3α,6α-Diacetoxy-12-oxocholanic acid	225-227		Diacetoxy-monooxo-cholanic acid	210
Me 3α,6α-Diacetoxy-12-oxocholanate	182-183			
3,6,12-Triketocholanic acid	221-223			198
Me ester	182–183			173–174
3,6,12-Trioxoallocholanic acid	239–241			234
Me ester	221–222			206–207

It is to be noted that the physical constants of 3α,6α,12α-trihydroxycholanic acid and its derivatives differ markedly from those of iso-cholic acid once described by Isaka and its corresponding derivatives. The physical constants of these substances are summarized in Table I.

Though there is no direct comparison because the sample of iso-cholic acid is not in our hand, it may be assumed that iso-cholic acid is not identical with 3α,6α,12α-trihydroxycholanic acid. Cholanic acid propyl ester (XI) (δ) was prepared from methyl 3,6,12-trioxocholanate by thioketalization with ethanedithiol and borontrifluoride-ether complex, reduction using Raney nickel, hydrolysis and esterification with *n*-propyl alcohol-hydrogen chloride. In the above experiment, allocholanic acid was not isolated. This compound, m.p. 56–57°, was identical with an authentic sample prepared from 3,12-dioxocholanic acid by Wolff-Kishner reduction followed by esterification with *n*-propyl alcohol-hydrogen chloride.

Allocholanic acid propyl ester was prepared from methyl 3,6,12-trioxoallocholanate by Huang-Minlon reduction followed by esterification as above. This compound, m.p. 103–104°, was shown to be identical with an authentic sample prepared from 3,6-dioxoallocholanic acid by the same method.

EXPERIMENTAL

Methyl 3α,6α,12α-Triacetoxycholanate (Vc)

(*a*) *By Huang-Minlon Reduction*—A mixture of 5 g. of methyl 3α,6α,12α-triacetoxy-7-oxycholanate (IIb), m.p 157–159°, 30 ml. of absolute ethanol and 5 ml. of hydrazine (98 per cent) was refluxed on a water bath for five hours, the ethanol and the excess of hydrazine were evaporated under reduced pressure and 20 ml. of benzene was added to the residue and evaporated*. To the residue 50 ml. of triethyleneglycol, 5 ml. of hydrazine and 4.5 g. of potassium hydroxide were added and the solution was heated at 190° for three hours. The solution was cooled to room temperature, poured into a large amount of water, acidified with diluted hydrochloric acid and extracted with ethyl acetate. The ethyl acetate solution was washed with water, dried and evaporated. The crude reaction mixture was methylated with diazomethane in methanol, followed by acetylation with acetanhydride and pyridine. The methyl ester acetate was chromatographed on 100 g. of alumina. From benzene-petroleum ether (3 : 2) eluate an oily product (1.0 g.) was obtained but was not examined further. From the next benzene-petroleum ether (3 : 2) eluate a crystalline product (10 mg.), was obtained showing m.p. 117-118°, after recrystallization from methanol-water. This was shown to be identical with methyl desoxycholate diacetate by mixed melting point. From benzene-petroleum ether (2 : 1–9 : 1) and benzene eluates a crystalline product (Vc) (2.43 g.; 50 per cent yield) was obtained. It was recrystallized from ether-petroleum ether and from methanol; m.p. 150–151°, $[\alpha]_D^{28}+58.6°$ (in dioxane).

Analysis Calcd. for $C_{31}H_{48}O_8$: C, 67.86; H, 8.82; CH_3CO, 23.52.
Found : C, 68.06; H, 9.05; CH_3CO, 23.41.

From benzene-ether and ether eluates an oily product was obtained but was not examined further.

(*b*) *By Hauptmann's Method*—A mixture of 5 g. of IIb, 15 ml. of ethanedithiol and 15 ml. of boron trifluoride-ether complex (BF_3; 47 per cent) was allowed to stand for 72 hours at room temperature, poured into ice water and extracted with ether. The

* In this reduction Vc was not isolated when the reduction was carried out in a usual manner and the yield decreased to 20–30 per cent using aqueous hydrazine hydrate.

ethereal solution was washed with water, dried and evaporated. The excess of ethanedithiol was removed by steam destillation, the residue was extracted with ethyl acetate and the ethyl acetate solution was dried and evaporated. A crystalline thioketal (III) was obtained when a small amount of ether was added to the residue. It was recrystallized from methanol; m.p. 214–215° (3.1 g), $[\alpha]_D^{23}+66.9°$ (in dioxane), λ_{max}^{Nujol} 5.75 μ, 5.78 μ, $\lambda_{max}^{SC_2}$ 5.76 μ.

Analysis Calcd. for $C_{33}H_{50}O_8S_2$: C, 62.04; H, 7.90; S, 10.03.
Found : C, 61.98; H, 8.01; S, 9.56.

The mother liquor was chromatographed on 20 g. of alumina. A small amount of crystal, m.p. 168 169°, was obtained from petroleum ether-benzene (3 : 2) eluate but was not examined further. From petroleum ether benzene and benzene eluates the thioketal (III) (0.6 g.) was obtained. To a solution of 1 g. of the thioketal (III) in 20 ml. of methanol, 10 g. of freshly prepared Raney nickel in methanol was added and the solution was refluxed on a water bath for 8 hours. The nickel was filtered off, the filtrate was evaporated to dryness and the residue was chromatographed on 20 g. of alumina. From petroleum ether-benzene (3 : 2–1 : 8) eluates a crystalline product (0.6 g.) was obtained; m.p. 117–118°. It was shown to be identical with methyl desoxycholate diacetate by mixed melting point and by comparison of each infrared spectrum. From benzene eluate a crystal (0.17 g.), m.p. 150–151°, was obtained, which was identical with the above mentioned Vc.

3α,6α,12α-Trihydroxycholanic Acid (*Va*)—500 mg. of Vc, m.p. 150–151°, was saponified with potassium hydroxide in methanol, the free acid was extracted with ethyl acetate and the ethyl acetate solution was washed, dried and evaporated. Only an amorphous product was obtained. All attempts to crystallize it were unsuccessful. $[\alpha]_D^{23}+17.8°$ (in dioxane).

Partial Acetylation of 3α,6α,12α-Trihydroxychclanic acid (*Va*) *and Its Methyl Ester* (*Vb*)

(*a*) *By Acetyl Chloride in Acetic Acid*—A mixture of 960 mg. of oily Va in 9 ml. of glacial acetic acid and 0.3 ml. of acetyl chloride was allowed to stand at room temperature for three days, poured into ice water and extracted with ether. The ethereal solution was washed with water, dried and evaporated. The product not being crystallized was exterified with diazomethane and the crude methyl ester was purified through 20 g. of alumina. From petroleum ether-benzene (3 : 2-1 : 9) eluates 630 mg. of crystal was obtained, which was shown to be identical with Vc by mixed melting point. From benzene eluate 97 mg. of needles (Vd), m.p. 152–154°, was obtained, which was recrystallized from ether-petroleum ether and methanol; m.p. 154–155°; $[\alpha]_D^{23}+20.3°$ (in dioxane); λ_{max}^{Nujol} 2.83 μ, 5.74 μ, 5.87 μ,; $\lambda_{max}^{CS_2}$ 2.76 μ, 5.76 μ.

Analysis Calcd. for $C_{29}H_{46}O_7$: C, 68.57; H, 9.30.
Found : C, 68.74; H, 9.15.

The mixed melting point of Vd with Vc was 125–140°

(*b*) *By Pyridine-Acetanhydride in Benzene*—A mixture of 700 mg. of Vb in 5 ml. of benzene, 0.8 ml. of pyridine and 0.8 ml. of acetanhydride was allowed to stand at room temperature overnight and poured into water. The benzene layer was separated and the water layer was extracted with ether. The combined organic layers were washed with diluted hydrochloric acid, water, diluted sodium carbonate solution and water, dried and evaporated. The residue was crystallized from ether-petroleum ether and 175 mg. of Vd, m.p. 154–155°, was obtained. The mother liquor was chromatographed on alumina and 550 mg. of Vc and 79 mg. of Vd were obtained.

Methyl 3α,6α-Diacetoxy-12-oxocholanate (*VIb*)—To a solution of 200 mg. of Vd in 4 ml.

of glacial acetic acid, 120 mg. of potassium chromate in 0.5 ml. of water was added and the solution was allowed to stand at room temperature for 16 hours, poured into water and extracted with ether. The ethereal solution was washed, dried and evaporated. 195 mg. of crystalline product (VIb) was obtained and recrystallized from methanol to needles, m.p. 182–183°; $[\alpha]_D^{23}$+54.9° (in dioxane); λ_{max}^{Nujol} 5.77 μ, 5.80 μ, 5.88 μ; $\lambda_{max}^{SC_2}$ 5.76 μ, 5.86 μ.

Analysis Calcd. for $C_{29}H_{44}O_7$: C, 69.03; H, 8.79.
Found : C, 68.86; H, 8.86.

3α,6α-Dihydroxy-12-oxocholanic Acid (VIa)—VIb was saponified in a usual manner and a crystalline free acid (VIa) was obtained and recrystallized from ethyl acetate-methanol to needles; m.p. 255–256° (with foaming); $[\alpha]_D^{23}$+62.4° (in dioxane); λ_{max}^{Nujol} 2.90 μ, 3.05 μ, 5.76 μ, 5.88 μ.

Analysis Calcd. for $C_{24}H_{38}O_5$: C, 70.90; H, 9.42.
Found : C, 70.90; H; 9.72.

Methyl 3α,6α-Diethoxycarbonyl-12α-hydroxycholanate (Ve) and Methyl 3α,6α-Diethoxycarbonyl-12-oxocholanate (VIc)—To a solution of 1.2 g. of Vb in 10 ml. of dioxane and 1.6 ml. of pyridine, 2 ml. of ethyl chlorocarbonate was added dropwise with stirring and cooling in ice-water, the solution was allowed to stand at room temperature for two hours and 30 ml. of water containing 1 ml. of concentrated hydrochloric acid was added. The reaction mixture was treated in a usual manner and 1.3 g. of oily product was obtained and purified through 3 g. of alumina; $[\alpha]_D^{23}$+39.2° (in 95 per cent of ethanol); $\lambda_{max}^{CS_2}$ 2.76 μ, 5.76 μ, 9.97 μ, 12.64 μ.

1 g. of this oily diethoxycarbonyl derivate (Ve) was oxidized with potassium chromate and acetic acid as mentioned above. 800 mg. of crystal (VIc) was obtained and recrystallized from methanol to plates; m.p. 160–161.5°; $[\alpha]_D^{23}$+56.8° (in dioxane); $\lambda_{max}^{CS_2}$ 5.76 μ, 5.86 μ, 9.90 μ, 12.65 μ.

Analysis Calcd. for $C_{31}H_{48}O_9$: C, 65.93; H, 8.57.
Found : C, 65.99; H, 8.70.

The free acid and the methyl ester diacetate were melted at 255–256° and 182–183° respectively. These compounds were shown to be identical with VIa and VIb respectively by mixed melting point.

α-Hyodesoxycholic Acid Methyl Ester Diacetate (VII)—A mixture of 62 mg. of VIb, m.p. 182–183°, 0.2 ml. of hydrazine hydrate (80 per cent), 5 ml. of triethyleneglycol and 140 mg. of potassium hydroxide was refluxed for an hour at 145°, evaporated until the temperature of the solution was raised to 200° and refluxed for three hours at 190–200°. Then the reaction mixture was cooled to room temperature, poured into ice water, acidified with diluted hydrochloric acid and extracted with chloroform. The chloroform solution was washed with water, dried and evaporated. Esterification with diazomethane, followed by acetylation gave 40 mg. of crystal, m.p. 90–96°, from methanol-water. Pure substance melted at 99–101°, was shown to be identical with the authentic specimen of α-hyodesoxycholic acid methyl ester diacetate by mixed melting point and by comparison of their infrared spectrum.

Analysis Calcd. for $C_{29}H_{46}O_6$: C, 70.99; H, 9.45.
Found : C, 70.92; H, 9.18.

Reduction of 12-Oxo Compounds VIa and VIb

(*a*) *By Lithium in Liquid Ammonia and Ethanol*—To a solution of 1.664 g. of VIa dissolved in 15 ml. of pure dioxane and 20 ml. of absolute ethanol, *ca.* 200 ml. of liquid

ammonia was added under cooling by dry ice-acetone and 1.5 g. of lithium cut into small pieces was added little by little with stirring. When the blue colour of lithium was disappeared, 11 g. of ammonium chloride was added, the ammonia was evaporated, 50 ml. of water was added and the solution was concentrated under reduced pressure. To the residue 50 ml. of water was added, the solution was acidified with diluted hydrochloric acid and the free acid was collected, washed with water and dried. The filtrate was extracted with ethyl acetate. The ethyl acetate residue was combined with the free acid and esterified with diazomethane. 1.695 g. of the methyl ester here obtained, was acetylated with pyridine and acetanhydride at 27° for 20 hours. 1.752 g. of the product was obtained and chromatographed on 60 g. of alumina. Each eluate was controlled to *ca.* 50 ml. From petroleum ether-benzene (2 : 1–1 : 2) (No. 1-31) eluates 1.452 g. of crystal, mp. 146–150°, was obtained, which was recrystallized from ether-petroleum ether-petroleum ether to plates having melting point 150–151°. This was shown to be identical with Vc by mixed melting point. From next petroleum ether-benzene (3 : 10–2 : 10) (No. 32–33) eluates 0.115 g. of crystal, m.p. 123–130°, was obtained, which was recrystallized from ether-petroleum ether to plates having melting point 129–132°*. This fraction was a mixture of Vc and Vd. The last eluates (No. 34–39) from benzene and benzene-ether having melting point 154–155° by recrystallization from ether-petroleum ether was identical with Vd. In this experiment 12β-hydroxyl derivative could not be obtained.

(*b*) *By Other Methods*—Reduction of VId was also attempted by catalytic reduction using Raney nickel in dioxane-methanol or using platinum oxide in acetic acid and by reduction of sodium borohydride in methanol, but in all cases the 12β-hydroxyl derivative was not obtained.

3,6,12-Trioxocholanic Acid (*VIIIa*) *and Its Methyl Ester* (*VIIIb*)—To a solution of 0.5 g. of Va dissolved in 5 ml. of glacial acetic acid, 0.5 g. of chromium trioxide dissolved in 2 ml. of glacial acetic acid and 1 ml. of water was added and allowed to stand for an hour at 15°. The solution was poured into 100 ml. of 2 *N* sulfuric acid and extract with dichloromethane. The dichloromethane solution was washed with water, dried and evaporated. The residue was crystallized from dichloromethane-ethanol and ethanol. 0.3 g. of crystal, m.p. 221–223°, was obtained : $[\alpha]_D^{31}$ −21.1° (in dioxane).

Analysis Calcd. for $C_{24}H_{34}O_5$: C, 71.61 ; H, 8.51.
Found : C, 71.70 ; H, 8.65.

Esterification of this acid with diazomethane gave a methyl ester, which was recrystallized from methanol to needles having melting point 182–183°.

Anatysis Calcd. for $C_{25}H_{36}O_5$: C, 72.08 ; H, 8.71.
Found : C, 72.00 ; H, 8.91.

3,6,12-Trioxoallocholanic Acid (*IXa*) *and Its Methyl Ester* (*IXb*)—0.15 g. of VIIIa dissolved in 10 ml. of 10 per cent sodium carbonate solution was heated on a water bath for an hour, cooled to room temperature, acidified with diluted hydrochloric acid, extracted with dichloromethane and the dichloromethane solution was washed, dried and evaporated. The residue was recrystallized from methanol-ethyl acetate ; m.p. 239–241° ; $[\alpha]_D^{31}$ +32.1° (in dioxane).

Analysis Calcd. for $C_{24}H_{34}O_5$: C, 71.61 ; H, 8.51.
Found : C, 71.29 ; H, 8.60.

* This compound was assumed to be the 12β-hydroxyl derivative and was reported at the Annual Meeting of the Japanese Biochemical Society in Fukuoka, November 1, 1956. But we correct here as it was proved from subsequent experiments that this compound was a mixture of Vc and Vd.

Methyl ester: m.p. 221–222° (from methanol);

Analysis Calcd. for $C_{25}H_{36}O_5$: C, 72.08; H, 8.71.
Found : C, 72.16; H, 8.56.

This allo-acid was also obtained from the mother liquor of the normal-acid (VIIIa).

Propyl Cholanate (*X*)—A mixture of 100 mg. of methyl 3,6,12-trioxocholanate (VIIIb), m.p. 182–183°, 0.5 ml. of ethane-dithiol and 0.5 ml. of boron trifluoride-ether complex was allowed to stand at room temperature for 48 hours, poured into ice water and extracted with chloroform. The chloroform solution was washed with water, dried and evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml. of dioxane and 10 ml. of methanol and reduced with Raney nickel, freshly prepared from 2 g. of nickel-aluminium alloy, by refluxing for 10 hours. The nickel was filtered off and the filtrate was evaporated to dryness under reduced pressure. The residue was saponified with potassium hydroxide in methanol and the free acid was esterified with 7 per cent hydrogen chloride in *n*-propanol. The crude *n*-propyl ester was purified through alumina with petroleum ether and crystallized from acetone-water with seeding. Analytically pure material was obtained after recrystallization from *n*-propanol: m.p. 56–57°; $[\alpha]_D^{22}+18.9°$ (in dioxane); reported (*8*) m.p. 56–57°.

Analysis Calcd. for $C_{27}H_{46}O_2$: C, 80.54; H, 11.52.
Found : C, 80.87; H, 11.46.

This compound was shown to be identical by mixed melting point and by comparison of each infrared spectrum with the authentic sample of *n*-propyl cholanate prepared from methyl 3,12-dioxocholanate.

n-Propyl Allocholanate (*XI*)—100 mg. of methyl 3,6,12-trioxoallocholanate (IXb), m.p. 221–222°, was reduced by Huang-Minlon method described above (V), esterified with 7 per cent hydrogen chloride in *n*-propanol. The *n*-propyl ester was recrystallized from *n*-propanol. m.p. 103–104°; $[\alpha]_D^{22}+21.0$ (in dioxane); reported m.p. 103–104° (*8*).

Analysis Calcd. for $C_{27}H_{46}O_2$: C, 80.54; H, 11.52.
Found : C, 80.87; H, 11.55.

This compound was shown to be identical by mixed melting point and by comparison of each infrared spectrum with the authentic sample of *n*-propyl allocholanate prepared from methyl 3,6-dioxoallocholanate by the same method.

SUMMARY

1. The conformation of the C-6 hydroxyl group of methyl 3α,6ζ,12α-triacetoxy-7-oxocholanate prepared from the 6α-brome compound by saponification was clarified as an equatorial (α).

2. 3α,6α,12α-Trihydroxycholanic acid and some of its derivatives were prepared and compared with iso-cholic acid and its derivatives.

The authors are grateful to Messrs. Ieki, Miyahara and Daikatsu for elemental analyses and to Messrs. Matsui and Tanaka for infrared spectral measurements.

REFERENCES

(*1*) Part X., Takeda, K., Kubota, T., and Matsui, Y., *Chem. Pharm. Bull.* (*Japan*) **6**, 437 (1958)

(2) Takeda, K., Komeno, T., and Igarashi, K., *Chem. Pharm. Bull.* (*Japan*), **2**, 325 (1954)
(3) Takeda, K., and Igarashi, K., *J. Pharm. Soc.* (*Japan*), (*Yakugaku-Zassi*), **76**, 867 (1956)
(4) Ohta, K., *Z. physiol. Chem.* (*Hoppe-Seyler's*), **259**, 53 (1939)
(5) Isaka, H., *Z. physiol. Chem.* (*Hoppe-Seyler's*), **266**, 117 (1940)
(6) Fieser, L. F., and Rajagopalan, S., *J. Am. Chem. Soc.*, **72**, 5530 (1950)
(7) Fieser, L. F., Herz. J. E., Klohs, M. W., Romers, M. A., and Utne, T., *J. Am. Chem. Soc.*, **74**, 3309 (1952)
(8) Windaus, A., and Bohne, A., *Ann. Chem., Liebigs*, **433**, 281 (1923)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

THE STRUCTURE OF CYTOCHROME C

VI. AMINO ACID COMPOSITION OF CYTOCHROMES C FROM BEEF-, HORSE- AND WHALE HEARTS, BAKER'S YEAST AND DESULFOVIBRIO DESULFURICANS

By KENJI TAKAHASHI, KOITI TITANI

(From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo)

and SHIGEKI MINAKAMI

(From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, March 9, 1959)

In the previous papers some descriptions were made on the relationship between the function and the structure of horse-heart cytochrome c, which had been investigated by the method of chemical and enzymatic modifications in the protein moiety of the pigment. For elucidating the function of cytochrome c based on the protein structure, comparative studies on the structure of cytochromes c from various sources would also be of great value. Along this line, one of the authors (S.M.) investigated in 1955 some physicochemical properties, which seemed to reflect the structure of horse heart and baker's yeast cytochromes c (*1*). In 1956 baker's yeast and different mammalian cytochromes c were crystallized by Okunuki and his co-workers. So the authors reported the re-examination of the above results and some further investigations (*2*, *3*)—amino acid composition by the DNP-method of Levy, c-terminal amino acid by the hydrazine method of Akabori and others—on mammalian heart and baker's yeast cytochromes c, which were obtained in crystalline or in the state as pure as crystalline. The data of amino acid composition obtained by the DNP-method, though easily obtainable, were not so exact to be regarded as a sound basis for further structural study and, therefore, it appeared necessary to re-examine the results by more exact methods.

In this paper, the authors report the amino acid composition of cytochromes c from various sources, which was determined by the method of Moore and Stein, as a primary step towards the elucidation of the chemical structure of cytochrome c, and also with an interest from the view-point of comparative biochemistry.

Until this investigation was undertaken, no exact analysis had been performed except that of beef-heart cytochrome c by Ehrenberg and Theorell (*4*). During the course of the study, amino acid analyses of horse-heart

and baker's yeast cytochromes c were reported by Nunnikhoven (*5*).

EXPERIMENTAL

Preparation of Cytochromes c—Cytochromes c from horse-, beef- and whale- hearts were extracted and purified by the method described in the previous paper (*6*). Baker's yeast cytochrome c was extracted with ammonium sulfate from ethyl acetate autolyzed cells, purified on an Amberlite IRC-50 column and crystallized according to the method of Hagihara *et al.* (*7*). Cytochrome c from *Desulfovibrio desulfuricans* (cytochrome c_3) was isolated from the acetone powder and purified on an Amberlite IRC-50 column according to the method of Ishimoto *et al.* (*8*).

Purity of Cytochromes c—The purity was examined by chromatography on an Amberlite IRC-50 column and paper-electrophoreses under various conditions, and every preparation of cytochromes c has been confirmed to be homogeneous.

The iron contents were also determined for each cytochrome c by the method of Saywell *et al.* (*9*), which were about 0.45 per cent for the three mammalian cytochromes c, 0.42 per cent for baker's yeast cytochrome c and 0.9 per cent for cytochrome c from *Desulfovibrio*. These were in good agreement with the value already reported for the most highly purified cytochromes c. The higher iron content of *Desulfovibrio* cytochrome c, just twice as high as other's, seemed to show that it contained two iron-porphyrin groups per molecule (*10*), which coincided with the observation that the absorbance at 550 mμ of reduced cytochrome c_3 was about twice as high as those from other sources on a molar basis. Or otherwise the molecular weight would be half that of mammalian and yeast cytochromes c.

The molecular weights were calculated from the iron contents to be 12,400 for the mammals', 13,300 for yeast and 12,400 for cytochrome c_3.

Among these, cytochromes from whale-heart and yeast were purified to crystallization and others to the highest purity as described above. The purity was also checked by measuring the molar extinction coefficients at 550 mμ of reduced cytochromes, which were in good agreement with those reported in the literature.

Amino Acid Analyses

Hydrolysis of Cytochrome c—About 0.2 μ moles of each cytochrome c (2 to 3 mg.) or several folds of the amount was weighed into a test tube, with the addition of 6 *N* glass-distilled hydrochloric acid (0.5 ml.). After sealing the tube, the hydrolysis was carried out in an oil-bath at 110° for 24 hours. The hydrolysate was dried in a vacuum desiccator over sodium hydroxide pellets to complete removal of hydrochloric acid, with occasional addition of small portions of water. The correct amount of each cytochrome was determined by measuring the weight after drying for 2 hours at 105° in an air-bath and cooling for 30 minutes at room temperature in a desiccator over calcium chloride. The weight was checked with the absorbance at 550 mμ of the reduced form of the dried cytochrome c.

Chromatography on a Column of Dowex-50X 4 (150 × 1.0 cm.)—Two samples of Dowex-50*X* 4, 200-400 mesh and −400 mesh, were tested at the preliminary stage and both gave similar results. In this experiment, the former was employed. The resin and the buffers were prepared according to the procedures described by Moore and Stein (*11*). The buffers were layered with liquid paraffin after boiling and stored in a cool place. Thiodiglycol, an antioxidant of methionine, was synthesized in our laboratory from sodium sulfide and ethylenechlorohydrin by the modified method of Meyer (*12*).

Preparation and operation of columns were carried out according to the procedure of Moore and Stein (*11*) with slight modifications. The dried hydrolysate from 2 to 3 mg. or so of cytochrome c dissolved in 1 ml. of 0.2 *N* sodium citrate buffer of pH 2.5,

was loaded on the column. In the preliminary experiment, the dried hydrolysate was dissolved in 2 ml. of 0.2 *M* phosphate buffer of pH 7.0 and kept to stand for 24 hours to oxidize cysteine, if any, to cystine. But it was found unnecessary, for neither cysteine nor cystine was detected on the chromatogram. The effluents were collected in 2 ml. fractions with an automatic fraction collector.

The chromatograms of three cytochromes—whale heart muscle, baker's yeast and *Desulfovibrio desulfuricans*—were shown in Fig. 1.

Chromatography on a Column of Amberlite IR-120 (15×1.0 cm.)—In the chromatography using a Dowex-50 column, high blank readings were obtained in the range, where ammonia, lysine and histidine emerged, which made it difficult to draw a correct base line and would be a source of errors in the calculation. Therefore the analyses of basic amino acids were also carried out with a column of Amberlite IR-120 (15×1.0 cm.) according to the procedure of Moore, Spackman and Stein (*13*), and the results of the Dowex-50 column were checked with those of the IR-120 column. The chromatogram of whale heart muscle preparation was shown in Fig. 2 as an example.

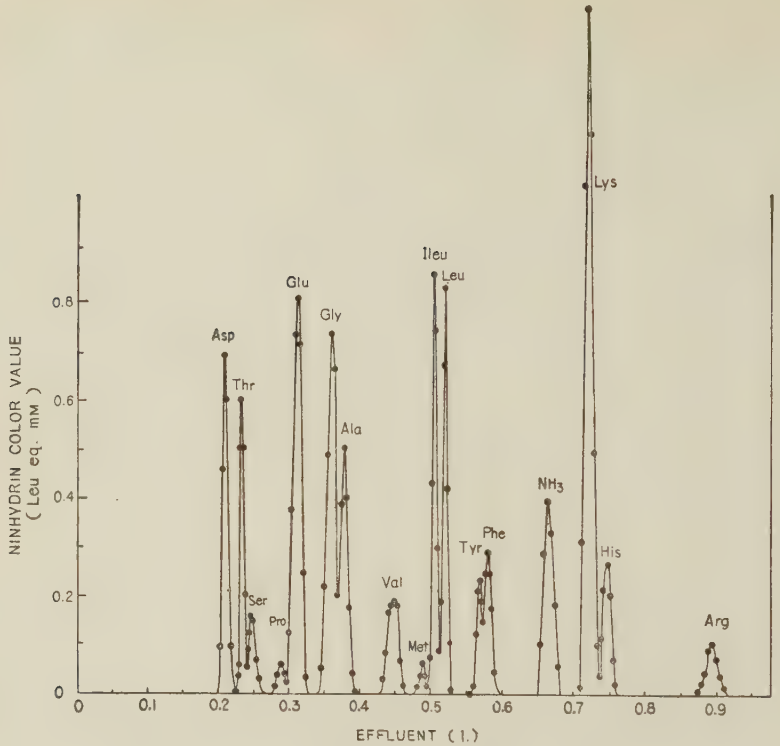
Procedures were similar to those of the Dowex-50 column. The resin used was sodium form and finer than 200 mesh. The dried hydrolysate from 1 to 2 mg. of each cytochrome c was dissolved in 1 ml. of 0.41 *N* sodium citrate buffer of pH 4.2 and loaded on to the column.

Determination of Amino Acids—The new photometric ninhydrin method of Moore and Stein (*14*) was employed with some modifications. The color development was examined at various concentrations of the ninhydrin solution and for several heating periods. The concentration of the ninhydrin solution was found to be decreased down to one-fourth of the original one with little influence on the color development when other conditions were equal. At the concentration lower than one-fourth of the original one, the color development was remarkably decreased and no linear relationship was obtained between the color development and the concentration of amino acids, even when the heating period was plolonged to 30 minutes or longer. So the ninhydrin solution of one-half the concentration of the original one was employed for economy of the expensive reagent. The diluted solution contained 10 g. of ninhydrin and 1.5 g. of hydrindantin per litre. Each aliquot of 0.5 to 1.0 ml. from the effluent fractions was analyzed with 1.0 ml. of the ninhydrin solution.

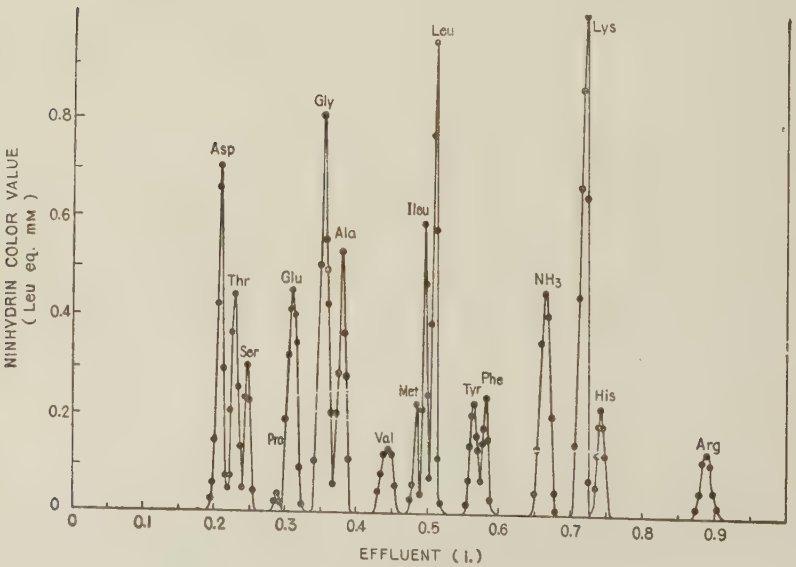
Amino acid composition of each cytochrome c was calculated from the concentrations of each amino acid determined as above, using the factor of the color development for each amino acid. Corrections of 16 per cent for serine and 8 per cent for threonine were made for the partial destruction during the acid hydrolysis. No correction was made for methionine for oxidation to methionine sulfoxide, since the latter was not clearly detected on the chromatograms. The values for ammonia were not corrected for the unknown amounts of ammonia accumulated from the reagents and evolved by the destruction of tryptophan, cysteine, serine, threonine *etc.* No analysis was performed by other methods for cysteine (or cystine) and tryptophan, which were not present in the acid-hydrolysates.

RESULTS AND DISCUSSION

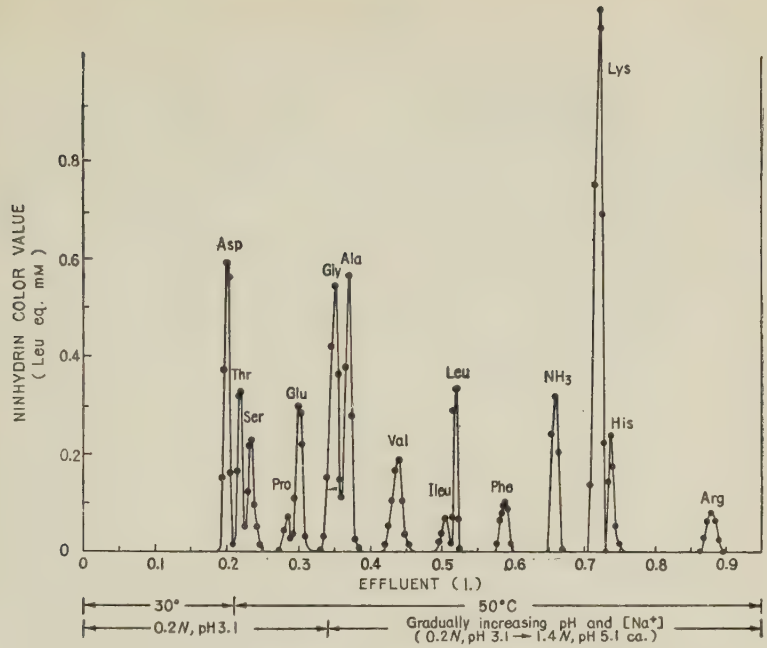
The results are summarized in Table I. The three mammalian cytochromes c were in good agreement with one another in amino acid composition. The results with beef-heart cytochrome c agreed quite well with those obtained by Ehrenberg and Theorell (*4*). We found somewhat



(a) Mammalian (whale heart) cytochrome c (3.76 mg.)



(b) Yeast cytochrome c (3.18 mg.)



(c) *Desulfovibrio* cytochrome c (3.00 mg.)

FIG. 1. Separation of amino acids on a column of Dowex 50X4.

Resin; Dowex-50X4 (200–400 mesh), Na form. Column; 150×1.0 cm. Mixing chamber; 600 ml. Initial flow rate; 8 ml./hour. Fraction; 2 ml./fraction. Load; hydrolysate of cytochrome c 2–4 mg. Elution solvents; sodium citrate buffers. Analysis; the ninhydrin method.

Sample; 1.0 ml; (or 0.5 ml.) from each fraction. Ninhydrin solution; 1.0 ml. (half the concentration of the original one) for each sample. Color development; at 100° for 15 minutes, with a galss-cap for each tube in a closed boiling water bath. Determination; Optical densities at 570 mμ (at 440 mμ for proline) were read after dilution with 4.5 ml. of 50 per cent ethanol.

more glutamic acid and phenylalanine and somewhat less tyrosine. Serine and threonine were successfully separated in our chromatography, to which Ehrenberg and Theorell had given a combined figure. The results with horse-heart and yeast cytochromes c were on the whole in good agreement with those obtained by Nunnikhoven (*5*). We found somewhat more aspartic acid, glycine and arginine with horse and more glycine and somewhat less proline and tyrosine with yeast.

Whale-heart cytochrome c was found to be very similar to beef- and horse-heart cytochrome c. It contained more serine and valine and somewhat less aspartic acid and methionine.

Cytochrome c from *Desulfovibrio desulfuricans* (cytochrome c_3) was found to be quite different from the mammalian and yeast cytochromes c. It contained much more serine, proline, alanine, valine, histidine and lysine and much less glutamic acid and isoleucine. Methionine and tyrosine were not

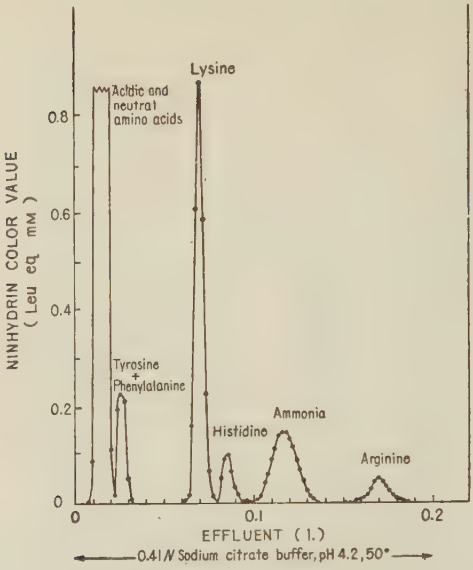


FIG. 2. Separation of basic amino acids on a column of Amberlite IR-120 (whale heart cytochrome c).

Resin; Amberlite IR-120 (finer than 200 mesh), Na form. Column; 15×1.0 cm. Initial flow rate: 12 ml./hour. Fraction; 2 ml./fraction. Load; hydrolysate of cytochrome c, 1.16 mg. Analysis; the ninhydrin method.

Sample; 2.0 ml. from each fraction.

found. The lack of tyrosine was in agreement with the finding that no appreciable absorption peak at 280 mμ was observed with this protein (*8*). These considerable differences would be related to the differences in the physiological functions. Yeast cytochrome could substitute mammalian one in a succinic oxidase system, while cytochrome c_3 could not. The finding (*10*, *15*) that cytochrome c_3 had a lower redox potential ($E'_0 = -0.205$ v.) than those of mammalian and yeast cytochromes c ($E'_0 = 0.25$ v.) is interesting in this connection.

In so far as observed, the high content of basic amino acids, especially of lysine, amounting to one-fourth of the total residues, is a common feature of every cytochrome c and the general pattern in amino acid composition is quite distinct from those of other basic proteins such as lysozyme. As to histidine, we found three moles per atom of iron for all the cytochromes tested, including cytochrome c_3 from *Desulfovibrio desulfuricans*. According to Theorell's view, two moles of the histidine residues are involved to form the hemochromogen structure on an iron atom. Our results might suggest the participation of the third molecule in the catalytic function.

Histidine and arginine were also determined previously (*2*) by the Pauly reaction and the Sakaguchi reaction respectively for horse-heart and yeast cytochrome c. Two and half moles of histidine and 2.7 moles of

TABLE I

Amino Acid Composition of Cytochromes c

(in moles of each amino acid residue per mole of cytochrome c)

Origin (mol. wt.) / Amino acids	Beef-heart (12,400)	Horse-heart (12,400)	Whale-heart (12,400)	Yeast (13,300)	*Desulfovibrio desulfuricans* (12,400)
Aspartic acid	9.2 (9.4)[1]	10.1	8.0	12.2	12.1
Glutamic acid	13.5 (11.9)	12.6	12.1	9.9	6.3
Threonine	8.4 } (8.5)	10.0	9.3	7.2	10.8
Serine	1.0 }	0.6	3.2	4.0	7.3
Proline	3.5 (3.9)	3.8	4.3	2.5	6.5
Glycine	16.5 (15.5)	14.1	16.4	16.3	15.6
Alanine	6.6 (6.6)	7.7	6.3	8.9	13.4
Valine	2.7 (3.3)	3.4	5.8	3.0	8.1
Methionine	1.6 (2)	1.6	0.6	1.7	0
Isoleucine	5.6 (6.2)	5.7	6.9	4.6	0.9
Leucine	6.0 (6.2)	6.6	6.8	7.2	4.3
Tyrosine	3.1 (3.8)	3.3	2.6	3.3	0
Phenylalanine	3.8 (3.3)	3.8	3.6	3.0	3.0
Lysine	18.7 (18.3)	17.7	17.7	15.1	23.4
Histidine	2.8 (3.1)	2.7	2.9	3.2	6.1
Arginine	2.9 (3.4)	2.4	2.6	3.1	1.8
Cystine	0 (0)	0	0	0	0
Cysteine[2]	(2) (2)	(2)	(2)	(2)	(4)
Tryptophan[2]	(1) (1)	(1)	(1)	(?)	(?)
Ammonia	8.5 –	8.2	10.0	12.9	8.3
Total residues	108.9 (108.4)	109.1	112.1	107.2	123.6

1) The figures given in the parentheses for beef-heart cytochrome c are the results obtained by Ehrenberg and Theorell (*4*).

2) No definite analysis was performed for cysteine and tryptophan and the values given in the parentheses are those assumed by analogy with the results of Ehrenberg and Theorell.

arginine per mole of the former and 2.8 moles of histidine and 3.2 moles of arginine per mole of the latter were found, which are in agreement with the results herein reported.

SUMMARY

1. The amino acid composition of cytochrome c from beef-, horse-, and whale-hearts, baker's yeast and *Desulfovibrio desulfuricans* was determined by the method of Moore and Stein. The three mammalian cytochromes c were in excellent agreement with one another, especially beef- and horse-

hearts cytochromes c being almost identical. Yeast cytochrome c was in close resemblance with the mammalians'. Cytochrome c from *Desulfovibrio* was strikingly different from those of the mammalians and yeast.

2. Three moles of histidine residues per atom of iron was found in all cytochromes c studied.

3. Neither cysteine nor cystine was detected in the acid-hydrolysate of every cytochrome c.

4. No tyrosine was found in cytochrome c from *Desulfovibrio.*

The authors express their thanks to Prof. S. Akabori and Prof. H. Yoshikawa for constant guidances during the course of the study and to Prof. F. Egami and Dr. M. Ishimoto for their interest and valuable discussions.

REFERENCES

(*1*) Minakami, S., *J. Biochem.*, **42**, 749 (1955)
(*2*) Minakami, S., Titani, K., and Ishikura, H., Symposium on Protein Structure, Fukuoka, October (1956)
(*3*) Titani, K., Ishikura, H., and Minakami, S., *J. Biochem.*, **44**, 499 (1957)
(*4*) Ehrenberg, A., and Theorell, H., *Acta. Chem. Scand.*, **9**, 1193 (1955)
(*5*) Nunnikhoven, R., *Biochim. et Biophys. Acta*, **28**, 108 (1958)
(*6*) Minakami, S., Titani, K., and Ishikura, H., *J. Biochem.*, **45**, 341 (1958)
(*7*) Hagihara, B., Horio, T., Yamashita, J., Nozaki, M., and Okunuki, K. *Nature*, **178**, 629 (1956)
(*8*) Ishimoto, M., Koyama, J., Yagi, T., and Shiraki, M., *J, Biochem.*, **44**, 413 (1957)
(*9*) Saywell, L., and Cunningham, G., *Ind. Chem. Anal. Ed.*, **9**, 67 (1937)
(*10*) Postgate, J., *Biochim. et Biophys. Acta*, **18**, 427 (1955); *J. Gen. Microbiol.*, **14**, 545 (1956)
(*11*) Moore, S., and Stein, W., *J. Biol. Chem.*, **211**, 893 (1954)
(*12*) *Organic Syntheses*, **12**, 69
(*13*) Moore, S., Spackman, D., and Stein, W., *Anal. Chem.*, **30**, 1185 (1958)
(*14*) Moore, S., and Stein, W., *J. Biol. Chem.*, **211**, 907 (1954)
(*15*) Ishimoto, M., Koyama, J., and Nagai, Y., *J. Biochem.*, **41**, 763 (1954)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

AMINO ACID DECARBOXYLASES OF PROTEUS MORGANII

II. SIMULTANEOUS FORMATION OF TWO INDUCED ENZYMES BY A SINGLE INDUCER

By SOHACHI ANDO

(*From the Institute of Food Microbiology, Chiba University, Narashino*)

(Received for publication, March 12, 1959)

During the course of investigation of biogenic amine formation by *Proteus morganii*, an organism causing allergic food poisoning, histidine decarboxylase was previously found to be inducible (*1*). Further studies on the formation of several other amino acid decarboxylases have revealed that they are also inducible under suitable conditions. Present paper deals with an interesting finding that some pair of these amino acid decarboxylases can be formed simultaneously in the presence of an inducer amino acid. The nature of the simultaneous formation of the enzymes seems to be different from that of the so-called "simultaneous adaptation" (*2*) or "successive adaptation" (3).

MATERIALS AND METHODS

L-Ornithine monohydrochloride was prepared from L-arginine by hydrolysis with saturated barium hydroxide (*4*). Other amino acids and α-ketoglutaric acid used in the present experiments were pure chemical grade of commercial origin. Pyridoxal phosphate was kindly supplied by Mr. T. Tanaka of Toa Eiyo Chemical Co., Ltd.

Proteus morganii was grown and adapted in media described previously (*1*). Estimation of decarboxylase activities was performed primarily by the method previously employed. In the case of glutamic decarboxylase, however, the method was modified as follows, since the enzyme was found to be very labile in intact cells. Washed cells of *Proteus morganii* were treated with cold acetone, dried in vacuum desiccator, and suspended in 0.1 *M* phosphate buffer (pH 5.0) to the density of 1.8 mg. dry weight of cells per ml. Activity of histidine decarboxylase was determined at pH 6.0 and of ornithine and glutamic decarboxylase at pH 5.0.

Preparation and activity measurement of transaminase were performed as follows: Washed cells of *Pr. morganii* grown in 300 ml. of basal synthetic medium for 18 hours were ground with emery at $-5°$ to $-10°$, suspended in 3 ml. of 0.1 *M* phosphate buffer (pH 8.0), then the emery and cell debris were removed by centrifugation at $25,000 \times g$ for 15 minutes at low temperature. The amount of glutamate formed by the enzyme preparation was made by the measurement of transaminase activity. The composition of reaction mixture of assay was of Gunsalus and Stamer (*5*). After 3 hours' incubation of the reaction mixture at 37°, the reaction was stopped by placing the mixture in a boiling water bath for 5 minutes and centrifuged. Prior to the determination of glutamate content in the mixture, excess α-ketoglutarate had to be removed, since acetone dried cells of *Escherichia coli*, used for glutamate assay as a glutamic decarboxylase preparation, con-

tained also transaminase which converts α-ketoglutarate to glutamate. One ml. of 2,4-dinitrophenylhydrazine dissolved in 2 *N* HCl (20 mg./ml.) was added into the supernatant of the reaction mixture, heated for 5 minutes, and the precipitates centrifuged off. Then excess phenylhydrazine contained in an aliquot of the mixture was further removed completely by repeated extraction with ethyl acetate and ether. Glutamate contained in the sample thus obtained was determined manometrically at pH 5.0 using acetone dried cells of *E. coli* strain No. 1, harvested from 1 per cent yeast extract-glucose broth.

RESULTS

Endogenous Activity and Non-induced Formation of Decarboxylases—In the present experiments, attention was focused on the formation of a group of enzymes decarboxylating histidine, ornithine and glutamate. The decarboxylase activities of cells grown in the medium, containing ammonium chloride, glutamate, cystine, glucose, two vitamins and salts, were found to be inactive on histidine, slightly active on ornithine and fairly active on glutamate (Table I). When the cells were incubated for 3 hours at 30° in the induction medium, which was similar to the growth medium except for the absence of glutamate, the formation of histidine and ornithine decarboxylases were found not to be demonstrable in the absence of the corresponding inducer amino acids, while glutamic decarboxylase was formed apparently without the addition of glutamate (Table II). If the corresponding inducer amino acids were added into the media, histidine and ornithine decarboxylases were formed and the rate of formation of glutamic decarboxylases increased. Thus these amino acid decarboxylases may be defined as inducible enzymes.

It should also be mentioned that during the course of 3 hour induction period, the cells increased in mass about 1.5 times the initial amount. The rate of growth of cells in the induction medium thus observed was found to de independent on the absence or presence of inducer amino acids.

Table I

Endogenous Activity of Decarboxylases in Proteus morganii

Decarboxylase	Endogenous activity ($\mu l.\ CO_2/hr./mg.$ *dry weight*)
Histidine	0
Ornithine	0.8
Glutamic acid	11.3

Inducer versus Decarboxylase Formation Relationship—In this series of experiments, the formation of more than one amino acid decarboxylases as induced by an inducer amino acid was tested. As already stated in the preceding paragraph, when an inducer amino acid was added, the appreciable formation of the corresponding amino acid decarboxylase was observed. Important is the fact that besides the formation of the corresponding decarboxylase

another decarboxylase is in some case formed simultaneously as clearly shown in Table II. Thus, when histidine was used as an inducer, enzymes decarboxylating histidine and ornithine but not glutamate were formed. This was also the case in ornithine, which induced the formation of enzymes decarboxylating ornithine and glutamate but not histidine. In the case of glutamate, however, only the formation of glutamic decarboxylase was accelerated leaving

TABLE II

Induced Formation of Amino Acid Decarbcxylases in the Absence or Presence of Various Amino Acids in Proteus morganii

Inducer	Decarboxylase activity ($\mu l.$ $CO_2/hr./mg.$ *dry wt.*)								
	Histidine			Ornithine			Glutamic acid		
	Incubation time in hour								
	0	1.5	3.0	0	1.5	3.0	0	1.5	3.0
No inducer	0	0	0	0.8	0.6	0.5	9.7	13.7	27.3
Histidine	0	8.0	12.9	0.8	1.1	2.4	9.7	15.8	26.3
No inducer	0	—	—	0.7	—	—	12.7	13.4	21.8
Ornithine	0	0	0	0.7	9.6	13.1	12.7	21.1	31.1
No inducer	0	—	—	0.8	—	—	11.5	12.8	22.6
Glutamate	0	0	0	0.8	0.8	0.7	11.5	13.1	27.6
Aspartate	0	0	0	0.8	0.7	0.7	11.5	16.9	33.5

TABLE III

Schematic Representation of Induced Formation of Amino Acid Decarboxylases in the Presence of Various Amino Acids

Inducer	Induced formation of amino acid decarboxylases		
	Histidine	Ornithine	Glutamic acid
Histidine	+	+	−
Ornithine	−	+	+
Glutamate	−	−	+
Aspartate	−	−	+

Plus and minus signs represent positive and negative formation of amino acid decarboxylases, respectively.

that of other decarboxylases unaffected so far as these three amino acid decarboxylases were concerned. Finaliy, aspartate was apparently found to be active in accelerating the formation of glutamic decarboxylase without effect on the other decarboxylases in question. Aspartic decarboxylase was not demonstrated in this organism either endogenously or inducibly.

To make the relationship between inducer and amino acid decarboxylase formation more perceivable, Table III is reproduced from Table II. Let us take into consideration of one couple of decarboxylases, such as, for example, histidine and ornithine decarboxylases. The former could be induced only by histidine and the latter by both histidine and ornithine. Thus, no cross induction was observed between these two enzymes as induced by the corresponding two amino acids. This was also found to be true in

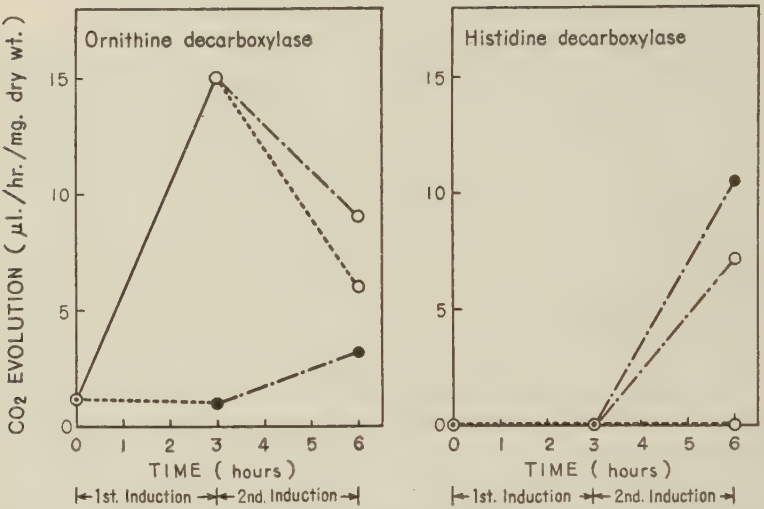


FIG. 1. Formation and loss of ornithine and histidine decarboxylases during the course of successive induction in the absence or presence of corresponding amino acids. ○: ornithine is added during 1st. induction period, ●: none is added during 1st. induction period, —: ornithine is present, —•—: histidine is present, - - -: none is added.

another couple, ornithine and glutamic decarboxylases. In order to analyze such relationship in more detail, the following experiment was carried out throwing a light on the formation of a pair of enzymes, histidine and ornithine decarboxylases. In this experiment, cells were first incubated in the absence or presence of ornithine (1st. induction), then the cells thus obtained were further incubated in the absence or presence of histidine (2nd. induction). After each of these successive inductions, the activities of ornithine and histidine decarboxylases were determined. The results are summarized in Fig. 1. As for the formation of ornithine decarboxylase during 1st. induction period, no activity increase was observed in the absence of ornithine, but when the cells thus treated were further incubated with histidine, apparent increase in ornithine decarboxylase (about 2 μl. CO_2/hour/mg. dry weight) was observed. In case the cells were first induced with ornithine, remarkable increase in ornithine decarboxylase activity was observed, but when the cells thus obtained were incubated in the absence of ornithine

during 2nd. induction period, ornithine decarboxylase activity was found to decrease appreciably. It should be noted, however, in the latter case the presence of histidine seemed to affect the course of decrease so as to cancel a part of decrement observed in the absence of the amino acid. The cancelled fraction of the decrement (about 3 μl. CO_2/hour/mg. dry weight) seems to correspond with the activity increment (about 2 μl. CO_2/hour/mg. dry weight) observed previously, *i.e.*, ornithine decarboxylase formation in the presence of histidine in cells previously treated without addition of ornithine. As far as histidine decarboxylase formation was concerned, the situation was much simpler than in the case of ornithine enzyme. No histidine decarboxylase was formed during the 1st. induction period in the absence or presence of ornithine, while when histidine was added to these cells, the formation of the enzme was quite apparent. The cause of the difference in the enzyme activities between cells treated previously with or without ornithine is still unsolved.

Enzymic Conversion of Amino Acids by Proteus morganii—The finding that a pair of decarboxylases is formed simultaneously suggests that the inducer amino acid added may be converted enzymatically to another amino acid which in turn induces the formation of corresponding amino acid decarboxylase. To assertain this possibility, conversion of a certain amino acid to another by *Proteus morganii* was tested.

The conversion of histidine to ornithine was tested in a system of the following composition: cell suspension (18 mg. dry weight of cells per ml.), 3 ml.; 0.1 *M* histidine, 0.5 ml.; and 0.1 *M* phosphate buffer (pH 6.0); 1.5 ml. After 3 hours of incubation at 30°, the mixture was heated for 5 minutes at 100° and centrifuged. The supernatant thus obtained was concentrated *in vacuo* and subjected to paperchromatography with a solvent system of *n*-butanol-acetic acid-water (4 : 2 : 1) or phenol saturated with water. The formation of ornithine, however, could not be detected under the present experimental conditions.

TABLE IV

Transaminase Activity in Proteus morganii

Amino acid	Glutamate formed (μ *moles*)		
	Exp. 1	Exp. 2	Exp. 3
Histidine	4.9	4.8	4.6
Ornithine	0.8	0.7	0.9
Aspartate	4.7	4.3	5.5

Reaction mixture contained; enzyme solution 0.5 ml., 25 μ moles of α-ketoglutarate, 0.1 μ mole of pyridoxal phosphate, 25 μ moles of amino acid in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer (pH 8.0).

In the next experiment, the conversion of histidine, ornithine and aspartate to glutamate by transaminase system in cell free extract of the

organism was tested using α-ketoglutarate as an amino group acceptor. The results are summarized in Table IV. It was thus found that asparatate and histidine were equally active in the transamination reaction to form glutamate, while ornithine only negligibly active.

DISCUSSION

The present observation of simultaneous formation of a pair of amino acid decarboxylases in the presence of an inducer amino acid seems to be similar to the phenomenon termed "simultaneous adaptation" (*2*) or "successive adaptation" (*3*). The phenomenon found by these investigators is defined that when an enzyme is adaptively formed against a certain substrate, other enzymes being capable of attacking those substrates, which are the intermediates on the metabolic pathway of the original substrate, are formed simultaneously. In the case of the present observation, however, the situation is somewhat different from the former. The enzyme which are subsidiary induced does not attack the product (amine and CO_2) of the enzyme primarily formed in the presence of an corresponding substrate (amino acid), but catalizes the similar reaction (decarboxylation), the substrate of which is different from the original one present in the system. Indeed, *Pr. morganii* adapted to the respective amino acids referred to in these experiments was found not to oxidize histamine, putrescine and γ-aminobutyric acid, which are the decarboxylation products of histidine, ornithine and glutamate, respectively. Therefore, the mechanism involved in the simultaneous formation of induced enzymes observed in the present experiments should be different from that of previous finding.

There is a possible explanation of the present phenomenon based on the substrate specificity of the induced enzyme. If the enzyme induced by an corresponding inducer amino acid has a broad specificity in decarboxylating amino acids, it may be able to decompose amino acids other than the one present in the system. Since the enzyme thus induced were found to be very labile, their purification were unsuccessful. Therefore, the examination of substrate specificity of the enzymes could not be demonstrated directly. It should, however, be mentioned that each amino acid decarboxylase hitherto found is usually accepted to catalize only one specific amino acid (*6*). Amino acid decarboxylases which are known to decarboxylate more than one amino acid are only those enzymes, such as glutamic acid (*7*)*, lysine (*8*) and tyrosine decarboxylase (*9*), which are able to attack either of corresponding amino acids and their hydroxy derivatives. It may be considered, therefore, that in the present observation the overlapping of the enzyme specificity range may not be the case in exhibiting the apparent simultaneous induction of these decarboxylase.

* Recently Umbreit and Heneage (*10*) and Miyaki, Hayashi, Wada and Matsumoto (*15*) showed indirect evidences that enzymes decarboxylating glutamic and hydroxyglutamic acids are different using intact cells of *Escherichia coli* as test organism.

It is probable to consider alternatively that when an amino acid is added to induce the formation of corresponding decarboxylase, a part of the amino acid may be converted by certain (constitutive) enzyme or enzyme system to another amino acid, which in turn induces the formation of the corresponding second decarboxylase. Although the enzyme activity converting histidine to ornithine or ornithine to glutamate could not be demonstrated with intact cells or cell free extract of *Proteus morganii*, the possibility of the interconversion of these amino acids (*11–13*) should be reserved. If this would be the case, the simultaneous induction of a pair of decarboxylases for histidine and ornithine or ornithine and glutamate in the presence of histidine or ornithine might be expected. The fact that the formation of glutamic decarboxylase occurred in the presence of aspartate seems to favor the possible mechanism described above, since the presence of glutamate-aspartate transaminase was apparently demonstrated. The proposed mechanism, however, seems not to cover all the cases of present findings. It was thus found that although the presence of glutamate-histidine transaminase was demonstrated in this system, no positive induction of glutamic decarboxylase was observed in the presence of histidine.

Finally, another alternative possible mechanism may be presented in the present phenomenon. As has been investigated by many investigators, β-galactosidase can be induced by several inducers which cannot be hydrolyzed by the enzyme (*14*). If this concept may be introduced into the consideration of the mechanism of the phenomenon, it will reasonably be explained that an amino acid induces the corresponding decarboxylase on the one hand and induces on the other hand different enzyme which decarboxylates another amino acid. It might indeed be considered that histidine and ornithine or ornithine and glutamate have analogous structure in carbon skeleton. If such an analogy with the induced formation of β-galactosidase would possibly be considered, the simultaneous induction of a pair of amino acid decarboxylases might be expected. Similar phenomenon is also well known in antigen-antibody reaction as "cross reaction".

Although it may still be premature to conclude the involvement of any of these mechanisms in the simultaneous induction of amino acid decarboxylases in *Proteus morganii*, one may be tempted to postulate the presence of alternative mechanisms as speculatively presented above in the simultaneous induction of enzymes.

SUMMARY

Induced formation of histidine, ornithine and glutamic decarboxylases in *Proteus morganii* was studied. It was found that a pair of these amino acid decarboxylases was formed simultaneously in the presence of an inducer amino acid. Histidine and ornithine enzymes were induced in the presence of histidine, and ornithine and glutamic enzymes in the presence of ornithine. Glutamate and asparate induced glutamic decarboxylase alone. The nature

of simultaneous induction of enzymes thus found seemed to be different from that of "simultaneous adaptation" or "successive adaptation" found previously. The possible mechanisms of the present finding were discussed.

The author wishes to thank Prof. K. Miyaki for his interest and encouragement, and Prof. T. Yanagita and Dr. M. Hayashi for their valuable advice and discussion.

REFERENCES

(*1*) Ando, S., *J. Biochem.*, **46**, 883 (1959)
(*2*) Stanier, R. Y., *J. Bacteriol.*, **54**, 339 (1947)
(*3*) Suda, M., Hayaishi, O., and Oda, Y., *Med. J. Osaka Univ.*, **2**, 21 (1950)
(*4*) Biochemical preparations, John Willey and Sons Inc., New York, Vol. **3**, pp 96 (1953)
(*5*) Gunsalus, I., and Stamer, J. R., "Transaminase in Bacteria", in "Method in Enzymology", edited by S. P. Colowick and N. O. Kaplan, Academic Press Inc. Publishers, New York, Vol. **2**, pp 170 (1957)
(*6*) Gale, E. F., *Advances in Enzymol.*, **6**, 1 (1946)
(*7*) Fowden, L., and Done, J., *Biochem. J.*, **53**, xxxi (1953)
(*8*) Linstedt, S., *Acta Chem. Scand.*, **5**, 486 (1951)
(*9*) Sourkes, T., Heneage, P., and Trana, Y., *Arch. Biochem. Biophys.*, **40**, 185 (1952)
(*10*) Umbreit, W. W., and Heneage, P., *J. Biol. Chem.*, **201**, 15 (1953)
(*11*) Vogel, H. J., *Proc. Natl. Acad. Sci. U. S.*, **39**, 578 (1953)
(*12*) Vogel. H. J., Abelson, P. H., and Bolton, E. T., *Biochim. et Biophys. Acta*, **11**, 584 (1953)
(*13*) Vogel, H. J., and Bonner, D. M., *J. Biol. Chem.*, **218**, 97 (1956)
(*14*) Cohn, M., *Bacteriol. Rev.*, **21**, 140 (1957)
(*15*) Miyaki, K., Hayashi, M., Wada, T., and Matsumoto, Y., *Chem. Pharm. Bull.*, **7**, 62 (1959)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

FORMATION OF β-GUANIDINOXYPROPIONIC ACID FROM L-CANAVANINE BY THE ACTION OF THE HEPATOPANCREAS OF MYTILUS EDULIS

By SEIICHIRO NAKATSU

(*From the Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka*)

(Received for publication, March 23, 1959)

It is well known that the hepatopancreas of a mollusc, *Mytilus edulis*, has the high activity of L-amino acid oxidase. Roche and co-workers (*1*) investigated the behavior of the hepatopancreas on L-arginine, and isolated α-oxo-δ-guanidinovaleric acid and γ-guanidinobutyric acid from incubation mixtures. Blaschko and co-workers (*2*) studied on the substrate specificity of L-amino acid oxidase from the same source, and obtained enzymatically α-oxo-γ-guanidinoxybutyric acid from L-canavanine as the 2,3-dinitrophenylhydrazone. The present author isolated a new guanidinoxy compound from a incubation mixture of L-canavanine with a suspension of the hepatopancreas, and the compound was identified as β-guanidinoxypropionic acid. In this paper, the identification, and chemical synthesis of β-guanidinoxypropionic acid are described.

MATERIALS AND METHODS

L-Canavanine Sulfate—L-Canavanine sulfate was prepared from the seeds of jack bean according to Fearon and Bell's method (*3*).

Enzyme Suspension—The mollusc, *Mytilus edulis*, was caught in Hakata Bay and dissected immediately. The hepatopancreas was separated from other tissues, weighed, and thoroughly ground with 1 ml. of cold water per each gram of the fresh tissue in a mixer. The mixture was then centrifuged, the supernatant was discarded, and the sediment was resuspended in the original volume of water. The mixture was recentrifuged, and the sediment was suspended in a small volume of 0.067 *M* phosphate buffer of pH 6.2. The suspension was dialysed against the same buffer for 24 hours in the cold, and was used as an enzyme suspension.

Paper Chromatography—Chromatograms were developed by the ascending technique on filter paper (Toyo-Roshi No. 52, 2×40 cm.) with a butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2), butanol-acetic acid-water (4 : 1 : 1), or *tert*-butanol-formic acid-water (15 : 3 : 2) mixture respectively. Guanidinoxy compounds on paper chromatograms were detected with a nitroprusside-ferricyanide reagent (*4*).

EXPERIMENTAL AND DISCUSSION

Isolation and Identification of β-Guanidinoxypropionic Acid

1 g. of L-canavanine sulfate was dissolved in a small volume of water,

and the pH of the solution was adjusted to about 6.2 with sodium hydroxide. An enzyme suspension containing 100 g. of the hepatopancreas was added, and the total volume was made up to 100 ml. with 0.067 *M* phosphate buffer of pH 6.2. After the addition of 10 ml. of chloroform and a few drops of octanol, the mixture was incubated at 35° under aeration with a slow stream of air. The experiment was followed by paper chromatography, and it was recognized that a spot corresponding to the new compound soon appeared and its amount increased gradually, while that of L-canavanine decreased. After 24 hours' incubation, the latter was no more detected. The mixture was then acidified to about pH 4.0 with acetic acid, and was heated for 10 minutes in a boiling water-bath. A dark green precipitate formed was filtered off, and the filtrate was passed through a column of a cation exchange resin, Amberlite IR-120 (H-form, 2×20 cm.). The resin was thoroughly washed with water, and the new compound adsorbed on the resin was eluted with 2 *N* ammonia water. (By this treatment using the resin, most of inorganic salts could be removed.) The eluate was concentrated to about 3 ml. under diminished pressure, and a solution of 1.2 g. of flavianic acid in 3 ml. of water was added. An orange-yellow precipitate was formed, which consisted mainly of the flavianate of the new compound. After being kept in a refrigerator overnight, the flavianate was collected and recrystallized twice from hot water.

The above flavianate was dissolved in 10 ml. of warm water, and the solution was made distinctly alkaline (pH 9.5) with a warm saturated solution of barium hydroxide. The barium flavianate precipitated was filtered off, and the excess of barium hydroxide in the filtrate was removed quantitatively with sulfuric acid. To remove the remaining trace flavianic acid, the solution was heated with a small amount of active charcoal for a while on a water-bath. The charcoal was filtered off, the filtrate was evaporated to about 1 ml. under diminished pressure, and 5 ml. of ethanol was added. A syrupy material was obtained, which crystallized after several days in a refrigerator. The crystals were collected and recrystallized from a small volume of hot water. The yield was 0.08 g. (16 per cent), the melting point (decomposition) 202°, and no rotation was observed with a polarimeter.

Analysis Calcd. for $C_4H_9O_3N_3$: C, 32.66; H, 6.17; N, 28.57 per cent.
Found : C, 32.39; H, 6.32; N, 27.95 per cent.

The compound gives a positive test with the nitraprusside-ferricyanide reagent and negative tests with ninhydrin and Sakaguchi's reagents. The R_f values on paper chromatograms with several different solvents and the melting point of the compound are identical with those of synthetic β-guanidinoxypropionic acid described below. It seems sure, therefore, that the isolated compound is β-guanidinoxypropionic acid. It may be possible that L-canavanine is first converted into α-oxo-γ-guanidinoxybutyric acid by the action of L-amino acid oxidase and the latter compound is then decarboxylated to β-guanidinoxypropionic acid with hydrogen peroxide which was formed enzymatically on the action of the oxidase (a flavin enzyme).

Synthesis of β-Guanidinoxypropionic Acid

β-Aminoxypropionic Acid Hydrochloride—β-Aminoxypropionic acid hydrochloride was synthesized according to the method reported by Hidy and co-workers (*5*).

β-Guanidinoxypropionic Acid—β-Guanidinoxypropionic acid was synthesized according to a modified method of that reported elsewhere by the present author (*6*).

To a solution of 0.142 g. (1 mM) of β-aminoxypropionic acid hydrochloride and 0.139 g. (1 mM) of *S*-methylisothiourea sulfate in 10 ml. of water was added 1 ml. (2 mM) of 2 *N* sodium hydroxide, and the solution was kept at room temperature for a week. The solution was then passed through a column of Amberlite IR-120 (H-form, 2×20 cm.), and the resin was washed completely with water. The β-guanidinoxypropionic acid adsorbed on the resin was eluted with 2 *N* ammonia water, and the eluate was concentrated to about 1–2 ml. under diminished pressure. A crystalline material appeared. After being kept in a refrigerator overnight, the crystals were collected and recrystallized from an adequate volume of hot 50 per cent ethanol. The yield was 0.10 g. (70 per cent), and the melting point (decomposition) was 205°.

Analysis Calcd. for $C_4H_9O_3N_3$: C, 32.66; H, 6.17; N, 28.57 per cent.
Found : C, 32.79; H, 6.15; N, 28.25 per cent.

The compound is easily soluble in hot water, slightly soluble in cold water, and insoluble in ethanol and ether.

SUMMARY

It was found that a new guanidinoxy compound was formed from L-canavanine by the action of the hepatopancreas of *Mytilus edulis*. The compound was isolated and identified as β-guanidinoxypropionic acid, which was also synthesized chemically.

The author wishes to thank Prof. Dr. S. Shibuya and Assistant Prof. Dr. N. Izumiya for their valuable advice.

REFERENCES

(*1*) Thoai, N., Roche, J., and Robin, Y., *Biochim. et Biophys. Acta*, **11**, 403 (1953)

(*2*) Blaschko, H., and Hawkins, J., *Biochem. J.*, **52**, 306 (1952); Blaschko, H., and Hope, D. B., *Biochem. J.*, **62**, 335 (1956)

(*3*) Fearon, W. R., and Bell, E. A., *Biochem. J.*, **59**, 221 (1955)

(*4*) Makizumi, S., *J. Chem. Soc. Japan* (*Pure Chem. Sec.*), **73**, 737 (1952)

(*5*) Hidy, P. H., Hodge, E. B., Young, V. V., Harned, R. L., Brewer, G. A., Phillips, W. F., Runge, W. F., Stavely, H. E., Pohland, A., Boaz, H., and Sullivan, H. R., *J. Am. Chem. Soc.*, **77**, 2345 (1955)

(*6*) Nakatsu, S., *The Memoirs of the Faculty of Science, Kyushu University, Series C, Chemistry*, **3**, 43 (1958)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

PREPARATION OF DL- AND D-CANAVANINE FROM THE L-FORM

By SEIICHIRO NAKATSU

(*From the Department of Chemistry, Faculty, of Science, Kyushu University, Fukuoka*)

(Received for publication, March 23, 1959)

L-Canavanine was first found in the seeds of jack bean by Kitagawa and Tomiyama (*1*), and is now known to be present in many kinds of leguminous plants (*2*). DL-Canavanine was synthesized through DL-canaline by Nyberg and Christensen (*3*), but the yield was low and no elementary analysis for the compound was given in their paper. In this work, DL-canavanine is prepared from the L-form by racemization with acetic anhydride, and furthermore, D-canavanine is prepared from the DL-form by enzymatic resolution using arginase.

EXPERIMENTAL

Arginase—The arginase used was a preparation from Nutritional Biochemicals Corporation in U.S.A., which contained 25–35 units of arginase per mg. The preparation was weighed and dissolved in a small volume of 0.067 *M* phosphate buffer of pH 7.7 containing 0.001 *M* manganous chloride, and the solution was used as an enzyme solution. The hydrolysis of L-canavanine by arginase was followed by the determination of urea according to Archibald's method (*4*), and the optimal pH for the reaction was found to be about 7.7.

Preparation of L-*Canavanine Sulfate* L-Canavanine sulfate ($C_5H_{12}O_3N_4 \cdot H_2SO_4 \cdot H_2O$) was prepared from the seeds of jack bean according to Fearon and Bell's method (*2*). The compound shriveled at 166° and decomposed 174°, and $[\alpha]_D^{15}$ was +19° (*c* 2.0, H_2O).

Preparation of DL-*Canavanine Sulfate*—To a chilled solution of 2.92 g. (10 mM) of L-canavanine sulfate and 1.06 g. (10 mM) of sodium carbonate in 15 ml. of water were added under vigorous strirring in several portions 1.22 g. (12 mM) of acetic anhydride and a solution of 1.38 g. (13 mM) of sodium carbonate in 10 ml. of water. The addition of the reagents required about 20 minutes, and the stirring was continued for 30 minutes thereafter. The solution was then acidified with 8.67 ml. (26 mM) of 3 *N* hydrochloric acid, and was evaporated to dryness under reduced pressure. The residue was extracted several times with absolute methanol, and the extracts were combined and evaporated under reduced pressure. A syrupy material, probably α-*N*-acetyl-L-canavanine, remained, which was dried in a desiccator.

The above syrup was dissolved in 20 ml. of glacial acetic acid (99.7 per cent), and 1.43 g. (14 mM) of acetic anhydride was added. The solution was heated for 2 hours on a water-bath, and then evaporated under reduced pressure. A syrupy material, probably α-*N*-acetyl-DL-canavanine, remained.

The above syrup was dissolved in 30 ml. of 2 *N* hydrochloric acid, and the solution was refluxed for 2 hours. The solution was then evaporated to dryness under reduced pressure, and the evaporation was repeated once more after the addition of 10 ml. of water. The residue was dissolved in 30 ml. of water, and a solution of 7.5 g. (20 mM) of flavianic acid in 30 ml. of water was added. The orange-yellow flavianate of DL-canavanine precipitated, which, after being kept in a refrigerator overnight, was collected and recrystallized twice from 50 ml. of hot water. The compound sintered at 180° and decomposed at 210°.

The above flavianate was dissolved in 100 ml. of warm water, and the solution was made distinctly alkaline (pH 9.5) with a warm saturated solution of barium hydroxide. The barium flavianate precipitated was filtered off, and the filtrate was acidified to about pH 3.0 with sulfuric acid. To remove the remaining trace of flavianic acid, the solution was heated with a small amount of active charcoal for a while on a water-bath. The barium sulfate and charcoal were filtered off, the filtrate was concentrated to about 5 ml. under reduced pressure, and 50 ml. of ethanol was added. A syrupy material precipitated. The supernatant was removed by decantation, and the syrup was dissolved in 5 ml. of 5 *N* sulfuric acid and precipitated again by the addition of 15 ml. of ethanol. The material crystallized after several days in a refrigerator. The crystals were collected, recrystallized from water (5 ml.)—ethanol (15 ml.), and dried over calcium chloride. The yield was 1.2 g. (44 per cent), and the melting point (decomposition) was 193°. No rotation was observed with a polarimeter, and only a half mole of urea was formed from one mole of the compound by the action of arginase.

Analysis Calcd. for $C_5H_{12}O_8N_4 \cdot H_2SO_4$:
C, 21.90; H, 5.14; N, 20.43; SO_4, 35.03 per cent.
Found :
C, 22.32; H, 5.34; N, 20.14; SO_4, 34.6 per cent.

Preparation of D-*Canavanine Sulfate*—In a small volume of water was dissolved 0.55 g. (2 mM) of DL-canavanine sulfate obtained above, and the pH of the solution was adjusted to about 7.7 with sodium hydroxide. An enzyme solution containing 50 mg. of the arginase preparation was added, and the mixture was incubated at 35° under toluene. The enzymatic hydrolysis was followed by the determination of urea, and it was found that, after 24 hours, one millimole of urea was formed and its amount no more increased. The incubation mixture was then acidified to about pH 3.0 with sulfuric acid, and was heated for 20 minutes on a water-bath. The protein coagulated was filtered off, and a solution of 0.74 g. (2 mM) of flavianic acid in 2 ml. of water was added to the filtrate. D-Canavanine precipitated immediately as the flavianate, while L-canaline and urea, which were formed from L-canavanine

by the action of arginase, remained in the solution. After being kept in a refrigerator overnight, the flavianate was collected and recrystallized twice from 5 ml. of hot water. The compound browned at 190° and decomposed at 215°.

The above flavianate was decomposed with barium hydroxide in the same manner as in the case of DL-canavanine. The resulting solution, which was free from flavianic acid and acidified with sulfuric acid, was concentrated to about 1 ml. under reduced pressure, and 5 ml. of ethanol was added. A syrupy material deposited. The supernatant was decanted off, and the syrup was dissolved in 0.5 ml. of 5 *N* sulfuric acid and reprecipitated by the addition of 1.5 ml. of ethanol. After several weeks in a refrigerator, the material crystallized. The crystals were collected, recrystallized from water (0.5 ml.)–ethanol (1.5 ml.), and dried over calcium chloride. The yield was 0.15 g. (51 per cent). The compound shriveled at 165° and decomposed at 172°, and $[\alpha]_D^{15}$ was −18° (*c* 2.0, H_2O).

Analysis Calcd. for $C_5H_{12}O_3N_4 \cdot H_2SO_4 \cdot H_2O$:
C, 20.55; H, 5.52; N, 19.17; SO_4, 32.87 per cent.
Found :
C, 21.01; H, 5.43; N, 19.20; SO_4, 33.4 per cent.

SUMMARY

1. DL-Canavanine sulfate was prepared from L-canavanine through α-*N*-acetyl derivatives of the L- and DL-forms. The yield was 44 per cent, and the melting point (decomposition) of the compound was 193°.

2. When DL-canavanine was subjected to the action of arginase, only the L-form was hydrolysed and the D-form was not attacked. From the incubation mixture, D-canavanine could be precipitated selectively as the flavianate, from which D-canavanine sulfate was prepared by the usual method. The yield was 51 per cent. The compound shriveled at 165° and decomposed at 172°, and $[\alpha]_D^{15}$ was −18° (*c* 2.0, H_2O).

The author wishes to thank Prof. Dr. S. Shibuya and Assistant Prof. Dr. N. Izumiya for their valuable advice.

REFERENCES

(*1*) Kitagawa, M., and Tomiyama, T., *J. Biochem.*, **11**, 265 (1929)
(*2*) Fearon, W. R., and Bell, E. A., *Biochem. J.*, **59**, 221 (1955); Bell, E. A., *Biochem. J.*, **70**, 617 (1958)
(*3*) Nyberg, D. D., and Christensen, B. E., *J. Am. Chem. Soc.*, **79**, 1222 (1957)
(*4*) Archibald, R. M., Ortiz, P., Stroh, E., and Bronner, J., *J. Biol. Chem.*, **157**, 507 (1945)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

ACTION OF TRYPSIN AND PAPAIN ON DERIVATIVES OF DIAMINOBUTYRIC ACID, ORNITHINE AND LYSINE

By NOBUO IZUMIYA, HIROKO OKAZAKI, ISAO MATSUMOTO and HIDEO TAKIGUCHI

(From the Laboratory of Biochemistry, Faculty of Science, Kyushu University, Fukuoka)

(Received for publication, March 26, 1959)

A previous communication from this laboratory has dealt with the mode of action of trypsin and papain on the homoylogous series of benzoylarginine amide and ester (*1*). This investigation has now been extended to include the homologous series of lysyl derivatives, namely the amides and methyl esters of N^{α}-benzoyl-L-ornithine and L-α-benzoylamino-γ-aminobutyric acid.

EXPERIMENTAL

Enzyme

Lyophilized acetyltrypsin and crystalline trypsin were the same materials as that previously described (*1*, *2*). Papain (PAP 5443) was crystalline suspension in 0.03 *M* cysteine from Worthington Biochemical Corp., U. S. A. The purity of papain was tested by its activity towards benzoyl-L-argininamide (L-BzArgAm). The enzyme N concentrations of papain were determined by measurements in Shimadzu spectrophotometer from a standard calibration curve at 275 mμ which was established by enzyme N analysis. At 39° and pH 6.0 (*M*/15 phosphate buffer) in the presence of 0.005 *M* L-cysteine and 0.001 *M* Versene, the enzyme hydrolyzed 0.05 *M* L-BzArgAm with a proteolytic coefficient of 0.64. The values of 1.0~1.2 were reported in the literatures (*3*, *4*).

Method

The procedure was about the same as that described before (*1*). In most cases, the hydrolysis of the substrates tested followed first order kinetics within experimental error. Proteolytic coefficients (*C*) were estimated from $C=k/e$, where $k=(1/\text{min.})\log(100/100-\%\ \text{hydrolysis})$ and e is the protein concentration in mg. of N per ml.

Synthesis of the Compounds

L-α-Amino-γ-carbobenzoxyaminobutyric Acid Methyl Ester Hydrochloride—L-α-Amino-γ-carbobenzoxyaminobutyric acid (12.6 g.) (*5*) was dissolved in 1 *N* methanolic HCl (360 ml.). The solution was left at room temperature overnight, and then evaporated *in vacuo*. The residue was dissolved in 1 *N* methanolic HCl, and the solution was repeatedly concentrated to dryness with addition of methanol after being left overnight. Treatment of the resulting syrup with acetone and ether yielded crystals. This was recrystallized from hot ethanol-ether; yield, 12.3 g. (81 per cent); m.p. 161–162°; $[\alpha]_D^{19}+21.6°$ (*c* 2, in water).

$C_{13}H_{19}O_4N_2Cl$ (302.8): Calcd. N 9.3
Found N 9.2

L-*α-Benzoylamino-γ-carbobenzoxyaminobutyric Acid Methyl Ester*—To a suspension of the above ester (7.6 g.) in chloroform (76 ml.) was added triethylamine (7.8 ml.). The compound went into solution immediately, and benzoyl chloride (2.9 ml.) was added to the solution under cooling. The mixture was allowed to react at room temperature overnight, washed with water, dilute bicarbonate solution, dilute HCl, and water; the organic layer was dried over Na_2SO_4, and concentrated *in vacuo* to yield a syrup which was crystallized by the addition of petroleum ether. This was recrystallized from hot ethyl acetate-petroleum ether; yield, 7.3 g. (79 per cent); m.p. 106°; $[\alpha]_D^{10}$ −27.2° (*c* 2, in ethanol).

$C_{20}H_{22}O_5N_2$ (370.4): Calcd. N 7.6
Found N 7.6

L-*α-Benzoylamino-γ-carbobenzoxyaminobutyramide*—The above ester (5.6 g.) was treated with methanolic NH, (110 ml.) in the usual manner (*6*). The product was recrystallized from hot methanol-ether; yield, 4.4 g. (83 per cent); m.p. 174°; $[\alpha]_D^{14}$ −13.2° (*c* 2, in dimethylformamide).

$C_{19}H_{21}O_4N_3$ (355.4): Calcd. N 11.8
Found N 11.7

L-*α-Benzoylamino-γ-aminobutyric Acid Methyl Ester Hydrochloride* (L-*BzDbaOMe HCl*)—The benzoylamino-carbobenzoxyaminobutyric acid methyl ester (3.7 g.) dissolved in 0.3 *N* methanolic HCl (36 ml.) was treated with dry hydrogen in the presence of palladium black. The filtrate was evaporated *in vacuo*, and the treatment of the resulting syrup with acetone and ether yielded crystals. This was recrystallized from hot methanol-ether; yield, 2.5 g. (91 per cent); m.p. 145–147°; $[\alpha]_D^{10}$ −31.5° (*c* 2, in water).

$C_{12}H_{17}O_3N_2Cl$ (272.7): Calcd. C 52.7, H 6.3, N 10.3
Found C 52.6, H 6.6, N 10.7

L-*α-Benzoylamino-γ-aminobutyramide Hydrochloride* (L-*BzDbaAm HCl*)—The benzoylamino-carbobenzoxyaminobutyramide (3.6 g.) was hydrogenated as described above. Yield, 2.3 g. (89 per cent); m.p. 186–187°; $[\alpha]_D^{10}$ −3.5° (*c* 2, in water).

$C_{11}H_{16}O_2N_3Cl$ (257.7): Calcd. C 51.2, H 6.3, N 16.3
Found C 49.8, H 6.3, N 16.7

N^α^-Benzoyl-N^δ^-carbobenzoxy-L-*ornithine Methyl Ester*—*N^δ^*-Carbobenzoxy-L-ornithine methyl ester hydrochloride (12.7 g.) (*7*) was benzoylated by the same procedure as described before. Yield, 10.1 g. (66 per cent); m.p. 105°; $[\alpha]_D^{10}$ −10.0° (*c* 2, in ethanol).

$C_{21}H_{24}O_5N_2$ (384.4): Calcd. N 7.3
Found N 7.3

N^α^-Benzoyl-N^δ^-carbobenzoxy-L-*ornithinamide*—The above ester (5.8 g.) was amidated in the usual manner. Yield, 4.8 g. (86 per cent); m.p. 195°; $[\alpha]_D^{14}$ +15.0° (*c* 2, in dimethylformamide).

$C_{20}H_{23}O_4N_3$ (369.4): Calcd. N 11.4
Found N 11.5

N^α^-Benzoyl-L-*ornithine Methyl Ester Hydrochloride* (L-*BzOrnOMe HCl*)—The benzoylcarbobenzoxyornithine methyl ester (3.8 g.) was hydrogenated in the same way as that for L-BzDbaOMe HCl. Yield, 2.6 g. (92 per cent); m.p. 142–143°; $[\alpha]_D^{10}$ −27.5° (*c* 2, in water).

$C_{13}H_{19}O_3N_2Cl$ (286.7): Calcd. C 54.3, H 6.7, N 9.8
Found C 54.0, H 6.8, N 9.5

N^α^-Benzoyl-L-*ornithinamide Hydrochloride* (L-*BzOrnAm HCl*)—This was made by the same procedure as that for L-BzDbaAm HCl. Yield, 87 per cent; m.p. 209–210°; $[\alpha]_D^{10}$ 0° (*c* 2, in water).

$C_{12}H_{18}O_2N_3Cl$ (271.7): Calcd. C 53.0, H 6.7, N 15.4
Found C 53.1, H 6.4, N 15.2

*N^{α}-Benzoyl-N^{ϵ}-carbobenzoxy-*L*-lysine Methyl Ester*—N^{ϵ}-Carbobenzoxy-L-lysine methyl ester hydrochloride (6.6 g.) (*8*) was benzoylated as described before. The product was obtained in crystalline form, though Bergmann *et al.* (*8*) had prepared it as an oily substance. Yield, 6.8 g. (85 per cent); m.p. 74–76°; $[\alpha]_D^{17} - 10.0°$ (*c* 2, in ethanol).

$C_{22}H_{26}O_5N_2$ (398.4): Calcd. C 66.3, H 6.6, N 7.0
Found C 66.1, H 6.6, N 6.9

*N^{α}-Benzoyl-*L*-lysine Methyl Ester Hydrochloride* (L-*BzLysOMe HCl*)—The above ester (2.0 g.) was hydrogenated in the usual manner. Despite repeated precipitation from methanol -ether, the substance could not be solidified, therefore the non-crystalline hygroscopic powder was used as a substrate; yield, 1.4 g.

*N^{α}-Benzoyl-*L*-lysinamide Hydrochloride* (L-*BzLysAm HCl*)—The crystalline compound was made as described in the literatures (*8*, *9*); m.p. 200–201°.

RESULTS AND DISCUSSION

Action of Acetyltrypsin—The optimum pH of acetyltrypsin for L-BzLysAm was determined, and the pH-activity curve was shown in Fig. 1, an optimum pH appearing near 8.7~8.8. The appearance of the pH-activity curve of L-BzLysAm is similar to that of L-BzArgAm (*1*). As shown in Table I, the enzyme failed to attack the amide derivatives of ornithine and diaminobutyric acid.

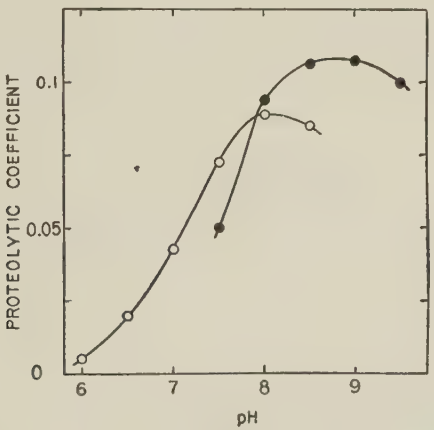


FIG. 1. The pH dependence of the hydrolysis of L-BzLysAm (0.01 *M*) by acetyltrypsin at 30°. ○, 0.1 *M* phosphate buffer. ●, 0.1 *M* Tris-HCl buffer.

Action of Trypsin—As shown in Table II, trypsin hydrolyzes very slowly the amide derivative of ornithine. In a previous communication, it was determined that trypsin hydrolyzed L-α-benzoylamino-γ-guanidinobutyramide at about 0.007 times the rate for L-BzArgAm (*1*). It was also shown that trypsin was not inhibited by the amide derivatives of ornithine or diaminobutyric

TABLE I

Hydrolysis of the Substrates by Acetyltrypsin

Substrate concentration, 0.01 *M*; pH 8.75 (0.1 *M* Tris-HCl buffer); temperature 30°.

Substrate	Enzyme concentration (*mg. protein N per ml.*)	*C*
L-BzDbaAm	0.35	0[1]
L-BzOrnAm	,,	0[1]
L-BzLysAm	0.057	0.11
L-BzArgAm	,,	0.19

1) No detectable hydrolysis in 5 hours.

acid in the hydrolysis of L-BzLysAm.

As seen from Table III, L-BzOrnOMe disappeared almost instantly at pH 7.8, although L-BzLysOMe was stable within 4 hours and L-BzDbaOMe disappeared gradually*. It was shown that the instant disappearance of L-BzOrnOMe was due to the formation of a six-membered lactam**. Therefore, BzOrnOMe could not be served as a substrate for an enzyme when Hestrin method is applied for the determination of the extent of hydrolysis***. It would be noteworthy that Barrass and Elmore have reported that the cyclic six- and five-membered lactams are produced from N^{α}-tosylornithine methyl ester and N^{α}-tosyldiaminobutyric acid methyl ester in methanolic NH_3, but seven-membered lactam from N^{α}-tosyllysine ester is less readily formed than the other two (*10*).

Brand *et al.* have reported that, in a preliminary manner, trypsin has no detectable action on poly-L-ornithine or benzoyl-L-ornithinamide (*11*). Recently, Erlanger has found that di- and tri-L-ornithine are resistant to the action of trypsin, papain and other enzymes (*12*). All the results obtained in this investigation and the literatures indicate the narrowness of the specificity of trypsin.

Action of Papain—Since no experiment concerning the optimum pH with

* It was also observed that the solution of BzOrnOMe in a buffer at pH 6.0 gave almost zero optical density by Hestrin method.

** 3-Benzoylamino-L-piperid-2-one was isolated as follows: To a solution of L-BzOrnOMe HCl (0.172 g.) in water (5 ml.) was added triethylamine (0.1 ml.). The solution was left overnight, and evaporated *in vacuo*. The crude crystals obtained was recrystallized from hot water. Yield, 0.105 g.; m.p. 173°; $[\alpha]_D^{18}+11.0°$ (c 2, in dimethylformamide). *Analysis* Calcd. for $C_{12}H_{14}O_2N_2$: C 66.0, H 6.5, N 12.8. Found: C 66.3, H 6.5, N 12.7.

*** Miss K. Kitagawa in this laboratory observed that L-α-benzoylamino-γ-guanidinobutyric acid ethyl ester, N^{α}-benzoyl-L-arginine methyl ester and N^{α}-benzoyl-DL-homoarginine ethyl ester were stable in a buffer at pH 7.8 within 2 hours at 30°.

TABLE II

Hydrolysis of the Substrates by Trypsin

Substrate concentration, 0.01 *M*; pH 7.8 (0.1 *M* phosphate buffer); temperature, 30°.

Substrate	Enzyme concentration (*mg. protein N per ml.*)	*C*
L-BzDbaAm	0.33	0[1]
L-BzOrnAm	,,	0.0009
L-BzLysAm	0.053	0.088
L-BzArgAm	0.028	0.16
L-BzLysOMe	0.000203	24[2]

1) No detectable hydrolysis in 5 hours.

2) The value given is extraporated initial constants, since decreasing values of *C* were observed.

TABLE III

Optical Density by Hestrin's Method of the Ester Compounds in the Absence of Enzyme

Compound concentration, 0.01 *M*; pH 7.8 (0.1 *M* phosphate buffer); temperature 30°. Certain amount of the incubation mixture was diluted with alkaline hydroxylamine, $FeCl_3$ was added, and the optical density of the red solution was read in a colorimeter. The detailed procedure is given in the communication (*15*).

Time (*min.*)	Optical density		
	L-BzDbaOMe	L-BzOrnOMe	L-BzLysOMe
0	0.29	0.04	0.63
30	0.28	0.05	0.62
60	0.27	0.05	0.63
120	0.21	0.04	0.63
240	0.09	0.05	0.63

a lysyl derivative for papain had been reported, measurements of the effect of pH on the amidase activity of the enzyme were made with L-BzLysAm as a representative substrate, and the pH-activity curve was shown in Fig. 2. For comparison, data for BzArgAm were included in Fig. 2. The optimum pH of the both cases appeared to be near 6 and 8. Smith *et al.* have studied in detail concerning the optimum pH of L-BzArgAm by papain (*3*, *13*).

In order to compare the hydrolytic rates of the amide substrates by papain, the values of C in various substrate concentrations and C_{max} at an optimum pH were determined. The results obtained are given in Table IV and V. The values of K_m and k_3 were determined by the use of plotting

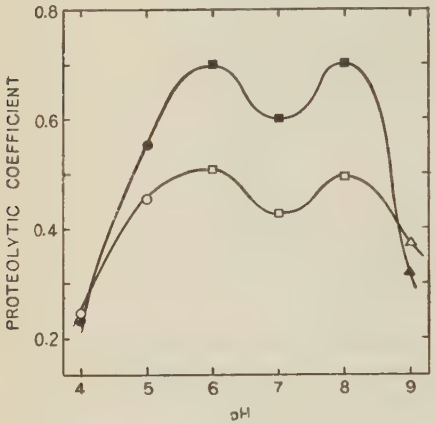


FIG. 2. The pH dependence of the hydrolysis of L-BzArgAm and L-BzLysAm by papain at 30° in the presence of 0.005 M cysteine and 0.001 M Versene. The substrate concentration was 0.01 M. ○, ●; M/15 acetate buffer. □, ■; M/15 phosphate buffer. △, ▲; M/15 Tris-HCl buffer.

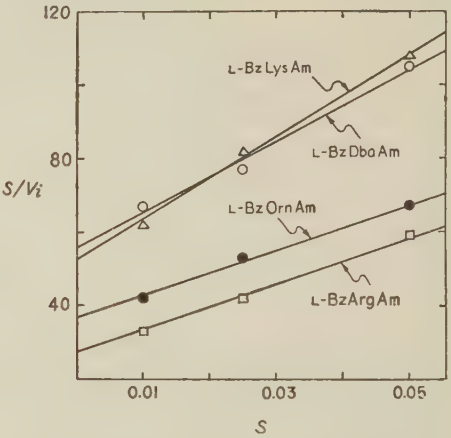


FIG. 3. The plots of initial substrate concentration S divided by initial velocity V_i *versus* S for the hydrolysis of the substrates at pH 6.0 and 30°. The enzyme concentrations were 0.0193 mg (L-BzOrnAm), 0.0125 mg. (L-BzLysAm), and 0.0184 mg. protein N per ml. (L-BzDbaAm and L-BzArgAm).

TABLE IV

Hydrolysis of the Substrates by Papain in Various Initial Substrate Concentrations

pH 6.0 (M/15 phosphate buffer) with 0.005 M cysteine and 0.001 M Versene; temperature 30°.

Substrate	Enzyme concentration (*mg. protein N per ml.*)	C		
		0.05 M	0.025 M	0.01 M
L-BzDbaAm	0.0184	0.22	0.31	0.35
L-BzOrnAm	0.0193	0.34	0.43	0.54
L-BzLysAm	0.0125	0.32	0.43	0.56
L-BzArgAm	0.0184	0.40	0.57	0.71
L-BzLysOMe	0.00216			4.3
L-BzArgOMe	0.00111			7.3

TABLE V

Kinetic Constants of the Substrate by Papain

pH 6.0; temperature 30°.

Substrate	K_m (M)	k_3[1]	C_{max}[2]
L-BzDbaAm	0.059	0.057	0.42
L-BzOrnAm	0.061	0.086	0.61
L-BzLysAm	0.049	0.073	0.66
L-BzArgAm	0.044	0.088	0.87

1) In mole/liter/minute/mg. protein N/ml.
2) $C_{max}=k_3/2.3\,K_m$.

method of Lineweaver and Burk (*6*), plots for the hydrolysis of the substrates being shown in Fig 3. The order of susceptibility to hydrolysis is listed as: L-BzArgAm > L-BzLysAm > L-BzOrnAm > L-BzDbaAm.

The qualitative study of Bergmann *et al.* has shown that crude papain hydrolyzes more rapidly L-BzArgAm than L-BzLysAm (*8*, *14*). It would be interesting to note that the ratios of the proteolytic coefficients of the homologous series of BzArgAm by papain (*1*) are similar qualitatively to that of BzLysAm.

SUMMARY

1. The amides and methyl esters of L-α-benzoylamino-γ-aminobutyric acid, N^{α}-benzoyl-L-ornithine and N^{α}-benzoyl-L-lysine have been synthesized.

2. The methyl ester of α-benzoylamino-γ-aminobutyric acid and N^{α}-benzoylornithine were unstable in a buffer of pH 7.8, five- and six-membered lactams being presumably formed.

3. Benzoylornithinamide is hydrolyzed very slowly by trypsin, although benzoyllysinamide is attacked easily by trypsin and acetyltrypsin. No hydrolysis of α-benzoylamino-γ-aminobutyramide by these enzymes was detected.

4. Papain hydrolyzes all the substrates tested.

REFERENCES

(*1*) Kitagawa, K., and Izumiya, N., *J. Biochem.*, **46**, 1159 (1959)
(*2*) Izumiya, N., and Uchio, H., *J. Biochem.*, **46**, 645 (1959)
(*3*) Kimmel, J. R., and Smith, E. L., *J. Biol. Chem.*, **207**, 515 (1954)
(*4*) Van Orden, H. O., and Smith, E. L., *J. Biol. Chem.*, **208**, 751 (1954)
(*5*) Izumiya, N., and Kitagawa, K., *J. Chem. Soc. Japan*, **79**, 65 (1958)
(*6*) Izumiya, N., and Yamashita, T., *J. Biochem.*, **46**, 19 (1959)
(*7*) Schumann, I., and Boissonas, R. A., *Helv. Chem. Acta*, **35**, 2237 (1952)
(*8*) Bergmann, M., Zervas, L., and Ross, W. F., *J. Biol. Chem.*, **111**, 245 (1935)

(*9*) Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130**, 81 (1938)
(*10*) Barrass, B. C., and Elmore, D. T., *J. Chem. Soc.*, 4830 (1957)
(*11*) Erlanger, B. F., *J. Biol. Chem.*, **224**, 1073 (1957)
(*12*) Brand, E., Plentle, A. A., and Erlanger, B. F., Abstracts, American Chemical Society, 116th meeting, Atlantic City, 27C (1949)
(*13*) Stockell, A., and Smith, E. L., *J. Biol. Chem.*, **227**, 1 (1957)
(*14*) Bergmann, M., and Fruton, J. S., *Advances in Enzymol.*, **1**, 63 (1941)
(*15*) Izumiya, N., and Yamashita, T., *J. Biochem.*, **46**, 337 (1959)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

THE ELONGATION AND DISSOCIATION OF MYOSIN B BY PYROPHOSPHATE

By TAI-ICHI NIHEI and YUJI TONOMURA

(From the Research Institute for Catalysis, Hokkaido University, Sapporo)

(Received for publication, March 27, 1959)

Since the size and shape change of the muscle structure protein, myosin B, by the addition of adenosine triphosphate (ATP) or inorganic pyrophosphate (PP) was first observed by Szent-Györgyi (*1*) and Needham *et al.* (*2*, 3), this reaction has been one of the subjects of worldwide investigations. The reaction mechanism remains, however, unsettled, and the two theories are being opposed to each other, because analysis of the myosin B system is very difficult owing to its polydispersity and extremely long shape. Szent-Györgyi (*1*), Weber and Portzehl (*4*), Gergely (*5*) and many other workers have reported experimental results indicating the dissociation of myosin B to myosin A and F-actin by the addition of ATP or PP, while Morales and his coworkers (*6*, 7) have concluded from light-scattering and ultracentrifugal data that the greater part of myosin B is elongated by ATP- or PP-addition. In order to clarify the reaction mechanism, it is considered to be necessary to study more systematically with myosin B prepared by a definite procedure. In the present paper the results obtained on the myosin B-PP system by the methods of light-scattering, ultracentrifugal separation and viscosity are reported, and in the subsequent paper (*8*) the results on the binding of PP to myosin B and A will be present. In general the data seem to confirm the elongation theory of Morales, though in details our conclusions seem to contradict his suggestion on the molecular mechanism of the elongation of myosin B.

METHODS

Preparation of Myosin B—The myosin B used was extracted from rabbit skeletal muscle with Weber-Edsall solution for 24 hours and purified by 3 or 4 times precipitation at 0.2 *M* KCl and dissolution at 0.6 *M* KCl. During these procedures the temperature was kept below 3°. All the protein solutions were stocked in a refrigerator maintained at 0° and were not allowed to stand longer than a week before use, unless otherwise stated. The stock solution was centrifuged at 14,000×*g* and at 0–1° for 1–2 hours in a Servall refrigerating centrifuge to remove gross impurities before use. This sample will be referred to as the "standard" one in the present paper. To measure the intensity of light-scattering and the viscosity, the "standard" solution (about 2 mg. protein/ml.) was first centrifuged at 25,000×*g* and at 0–1° for 2.5 hours in a Hitachi preparative ultracentrifuge (Model 40P), then diluted by the solvent filtered through a Millipore filter.

Light-Scattering Measurements—The light-scattering measurements of 0.6 *M* KCl solution

of myosin B (pH 7.2) were made in a Brice-Phoenix photometer (Model 1000D) (*9*) at room temperature. A light having a wavelength of 5,460 Å from the mercury arc was used. A cylindrical cell (T-101) was used in the angular measurements. The angles (θ) at which the measurement was perused were from 23 to 135°. The angular envelope of the cell was checked by measuring the fluorescence of a 0.2 per cent fluorescein solution. To correct the back reflection, the equation of Oth *et al.* (*10*)

$$(I_\theta)_{\text{real}}=(I_\theta)_{\text{exp}}-0.045\,(I_{180-\theta})_{\text{exp}}$$

was applied. Here $(I_\theta)_{\text{real}}$ and $(I_\theta)_{\text{exp}}$ are the real and the experimental intensities of the scattered light at the angle θ respectively. The refractive index increment (dn/dc) which is required for the calculation of the constant $K=2\pi^2 n_0^2(dn/dc)^2/(N_0\lambda^4)$, where n_0 is the refractive index of the solvent, λ the wavelength of the incident light, N_0 Avogadro's number, was determined on a Brice-Phoenix differential refractometer (*11*) at room temperature, using the same wavelength of light as the one in the scattering measurements. A value of $dn/dc=0.200$ was adopted in the presence and the absence of PP. To evaluate the "light-scattering" average molecular weight ($<M>_l$) (see "Discussion") Kc/I_θ was plotted against $\sin^2\theta/2$ (*12*).

Ultracentrifugal Separation—The ultracentrifugal separation was performed following the procedure of A. Weber (*13*): myosin B solution in 0.6 *M* KCl and 0.04 *M* Tris-maleate buffer, pH 7.2–7.4, was ultracentrifuged in polyethylene bottles at a mean centrifugal force $98{,}000\times g$ for 3 hours at 3–5° in a Hitachi preparative ultracentrifuge. The concentration of protein ranged from 0.8 to 3 mg./ml. After the ultracentrifugation the protein was pipetted out from an appropriate layer in the polyethylene bottle and the protein concentration was determined from the intensity of light-absorption at 280 mμ. The light-absorption was standardized by the nitrogen value determined by the micro-Kjeldahl method, correcting the absorption of the solvent mainly due to Tris-aminomethane.

Viscosity Measurements—A viscometer was constructed by the method of Claesson and Lohmander (*14*). It consisted of a 9.967 cm. length of capillary of radius 0.0318 ± 0.0002 cm. with wide cylindrical tubes of radius 1.3 cm. The movement of the meniscus in the cylindrical tube was followed by a micrometer. The maximum shearing stress (τ_{RS}) is given by

$$\tau_{RS}=PR/2\,l,$$

where R and l are the radius and the length of the capillary respectively and P is the pressure difference. Then, the relative viscosity (η_{rel}) of a solution may be calculated as a function of the mean velocity gradient ($<G>$) according to the following equations (*15*):

$$<G>=6F(\tau_{RS})+2\tau_{RS}\frac{dF(\tau_{RS})}{d\tau_{RS}},$$

where

$$F(\tau_{RS})=V/2\pi R^3 t_s,$$

and

$$\eta_{\text{rel}}=\frac{4/t_0}{3/t_s+1/t_0\dfrac{dl/t_s}{dl/t_0}},$$

where t_s and t_0 are the efflux times of a solution and a solvent of the volume V respectively. Our apparatus provided measurements in the range of 10–1,000 sec.$^{-1}$ mean gradients. The measurements were made at 20°.

Other Procedures—Protein concentrations were determined by the standard micro-Kjeldahl method, the factor 6 being used for converting nitrogen value to protein (*16*). pH was determined by a Beckman model G instrument.

RESULTS

Light-Scattering—Over the range of protein concentration (c) from 0.5 to 0.05 mg./ml., I_θ/c was independent of c at all angles measured. Accordingly the angular distribution of light scattered by myosin B was usually determined at a fixed protein concentration, about 0.05 mg./ml. It was also observed that I_θ was constant over the range of pH from 6.0 to 9.5 and it maintained its value unchanged for longer than 2 hours.

Fig. 1 illustrates a typical example of the reciprocal reduced intensity plots and Fig. 2 shows the scattering envelopes, *i.e.* the reciprocal angular distribution functions ($P^{-1}(\theta)$) as a function of $\sin^2\theta/2$, for myosin B. The "light-scattering" average molecular weight ($<M>_l$) and radius of gyration ($<r^2>_l^{1/2}$) of four samples are listed in Table I, errors of these values being ±5-10 per cent. It is obvious from these Figs. and Table that, while $<M>_l$ distributes over a wide range from 4.6 to 18.2×10^7 g., $P^{-1}(\theta)$ is almost constant. The $P^{-1}(\theta)$ plot curves gently downward and it is different distinctly from the one for monodisperse rod, as illustrated in Fig. 1.

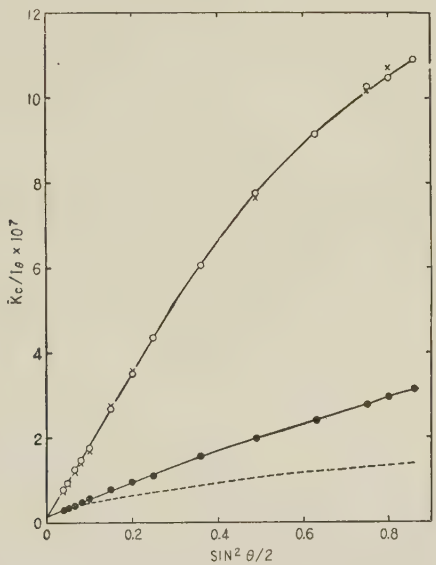


FIG. 1. The reciprocal reduced intensity plot for myosin B (sample No. VIII 1).

0.6 M KCl, 1×10^{-3} M $MgCl_2$, pH 7.2, 23°. Control, ●; 0.5×10^{-3} M PP, ○; 0.5×10^{-3} M Salyrgan, ×. The dotted line represents the reciprocal reduced intensity plot for a monodisperse rod.

When $0.5\sim1\times10^{-3}$ M PP was added to myosin B in the presence of 10^{-3} M $MgCl_2$, the maximum decrease of intensity of scattered light was observed (I_{90} fell to about 35 per cent of the original value), I_θ being unchanged by

increasing concentration of PP from 1 to 10×10^{-3} *M*. As indicated in Fig. 1, after the addition of PP the slope of the plot, Kc/I_θ $-\sin^2\theta/2$, increased remarkably maintaining essentially the same intercept, showing thereby that

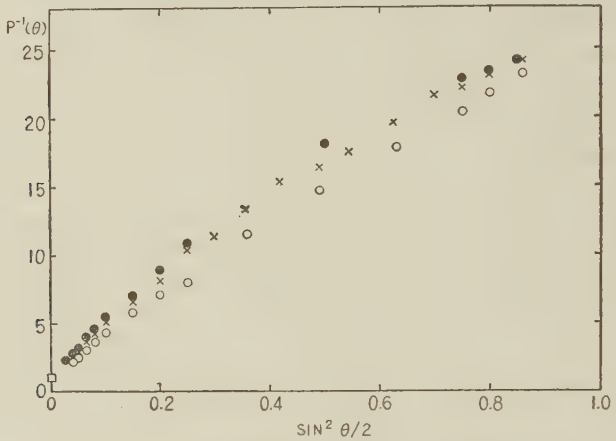


FIG. 2. The reciprocal scattering envelope for three samples. Prep. No. VIII 1, ○; No. VIII 2, ●; No. VIII 4, ×.

TABLE I

The Effect of 10^{-3} M PP on the Light-Scattering Average Molecular Weight and Radius of Gyration of Myosin B

Intensity of scattered light was measured in 0.6 *M* KCl and 10^{-3} *M* $MgCl_2$ pH 7.2 and at 23°.

Prep. No.	Before PP		After PP	
	$<M>_l$ × 10^{-7} g.	$<r_2>_l^{1/2}$ × 10^{-2} Å	$<M>_l$ × 10^{-7} g.	$<r^2>_l^{1/2}$ × 10^{-2}Å
VIII 1	6.65	26.8	6.65	53.6
VIII 2	18.2	31.0	18.2	67.8
VIII 3	4.6	28.2	4.6	45.7
VIII 4	9.1	29.5	—	—

the molecular size was increased greatly while the molecular weight remained essentially constant (see "Discussion"). These results confirm the one reported previously by Morales *et al.* (*6*, *7*). Furthermore, 5×10^{-3} *M* Salyrgan showed the same effect on the size and shape of myosin B as PP did (Fig. 1).

After storage for more than 7 days, adenosine triphosphatase (ATPase)

activity remained constant, but the light-scattering measurements of the "standard" solution indicated a great increase in $<M>_l$ during storage, though its exact value could not be obtained because of incomplete removal of dust. When the solution was centrifuged for 2 hours at $35{,}000 \times g$, $<M>_l$ was greatly diminished, indicating removal of aggregates which was produced during storage, and on the addition of PP $<M>_l$ fell, in extreme case, to about 60 per cent of the original, as shown by the representative data in Table II. Since Gergely (*5*) has reported that, after ultracentrifugation at extremely low concentration, $<M>_l$ decreases on the addition of PP, our "standard" sample was ultracentrifuged for 3 hours at $35{,}000 \times g$ at protein concentration of 0.05 mg./ml. Then it was found that $<M>_l$ decreased on PP-addition, though in less degree than Gergely's observation, as indicated in Table II and Fig. 3.

TABLE II

The Effect of 10^{-3} M PP on the Light-Scattering Average Molecular Weight and Radius of Gyration of Myosin B Obtained by Ultracentrifugation After Storage of 7 Days ar Dilution to 0.05 mg./ml.

Intensity of scattered light was measured in 0.6 *M* KCl and 10^{-3} *M* $MgCl_2$ at pH 7.4 and 20°.

Prep. No.	Before PP		After PP	
	$<M>_l$ × 10^{-7} g.	$<r^2>_l^{1/2}$ × 10^{-2} Å	$<M>_l$ × 10^{-7} g.	$<r^2>_l^{1/2}$ × 10^{-2} Å
VIII 3 (ultracentrifugation after storage)	1.5	15	0.95	25
VIII 5 (ultracentrifugation after dilution)	3.45	17	1.96	18
VIII 6 (ultracentrifugation after dilution)	4.76	17	3.85	21

Ultracentrifugal Separation—When the "standard" solution of myosin B was ultracentrifuged at $98{,}000 \times g$ for 3 hours, the solution was separated into a small quantity of pellet, turbid lower layer, whose volume was one third of the total, and upper transparent layer, as already reported by A. Weber (*13*). After pipetting out the supernatant of the second quarter layer from the surface, the concentration of the protein (denoted as the light component) was determined and the total quantity was calculated to be 11.4 (±3.9) per cent (Table III). The intensity of light scattered from the supernatant was lower than 5 per cent of that of the original solution at all angles measured and it did not change on the addition of PP, while the light scattered by

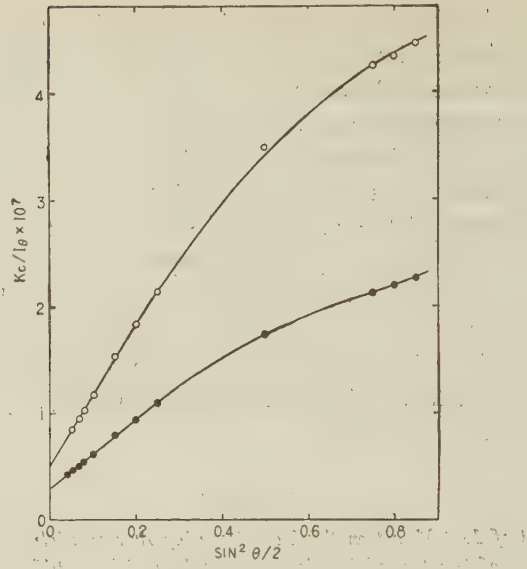


FIG. 3. The reciprocal reduced intensity plot for preparation No. VIII 5 after ultracentrifugation at 0.05 mg./ml.0.6 *M* KCl, $10^{-3}M$ $MgCl_2$, pH 7.4, 20°. Control, ●; $10^{-3}M$ PP, ○.

TABLE III

The Ultracentrifugal Separation of Light and Heavy Components of Myosin B Preparation

Myosin B was ultracentrifuged at 98,000 × *g* for 3 hours in 0.6 *M* KCl and $10^{-3}M$ $MgCl_2$ at pH 7.2–7.4 and at 3–5°.

Prep. No.	Before PP		After PP	
	Light comp. (*per cent*)	Heavy comp. (*per cent*)	Light comp. (*per cent*)	Heavy comp. (*per cent*)
VIII 1	18.0	82.0	41.0	59.0
VIII 2	12.0	88.0	24.3	75.7
VIII 7	9.3	90.7	33.0	67.0
VIII 8	6.3	93.7	23.1	76.9
VIII 9	10.0	90.0	30.2	69.8

the turbid layer showed typical responce to PP by increasing $< r^2 >_t^{1/2}$ with constant $<M>_l$. As will be described in a future paper, the specific activity of ATPase of the pellet was almost identical to that of the heavy component

(*i.e.* total protein minus light component) in the turbid layer, the former being 2.3 and the latter 2.5 μg. P/minutes/mg. protein in the presence of 0.6 M KCl and 3×10^{-3} M $CaCl_2$ and at pH 7.0 and 20°.

On the other hand, when the myosin B solution was ultracentrifuged in the presence of 10^{-3} M PP and 10^{-3} M MgCl, it was separated into precipitate and water-clear layer. The concentration of protein of the supernatant of the second quarter layer from the surface was found to be almost identical to the one of the third quarter layer, thus showing that the concentration of the light component was determined correctly. In sharp contrast with A. Weber's finding (*13*) it was observed that the total quantity of the light component was as small as 30.3 (±6.6) per cent of the total, as shown in Table III. It was found that the result obtained at 30° was almost the same as that at 3°, and that a small quantity of pellet (5–10 per cent of the total), which was formed by centrifugation of the "standard" solution at $25{,}000\times g$ was completely non-dissociable, as in the presence of PP the content of the light component of the "standard" preparation was identical to the one centrifuged beforehand at $25{,}000\times g$. The cause of discrepancy of our result with A. Weber's is not yet clear. This point may be a subject of a future research.

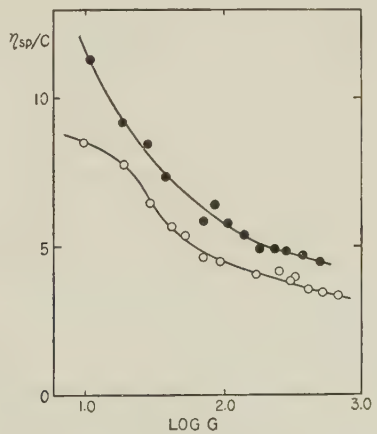


FIG. 4. The reduced viscosity of myosin B (sample No. VIII 10) as a function of velocity gradient in the presence (○) and the absence of $10^{-3}M$ PP (●).
0.6 M KCl, 10^{-3} M $MgCl_2$, pH 6.7, 20°, 1.08 mg./ml.

Viscosity—The reduced viscosity (η_{sp}/c) of myosin B was observed to be independent of c at all velocity gradient measured, when c was lower than 1 mg./ml. The reduced viscosity decreased with increase of gradient, but the dependence on $<G>$ fluctuated greatly from one preparation to another; for example the reduced viscosity of one preparation reached to a constant value, 15 in units of 100 ml./g., at low gradient, but in the case of the sample No. VIII 10 illustrated in Fig. 4 its reduced viscosity did not exhibit a tendency to approach to a definite value even at the lower gradient.

As shown in Fig. 4, the reduced viscosity decreased on the addition of PP but not remarkably at low gradient; for instance, at gradient 45 sec.$^{-1}$ it fell to 77 per cent of the original. In the presence of PP, the reduced viscosity of sample No. VIII 10 increased with decrease of gradient and the intrinsic viscosity at zero gradient ($[\eta]_0$) was found to be 8.8 (100 ml./g.).

DISCUSSION

To interpret the results obtained by the light-scattering method, it is necessary to assign the type of average, as Benoit *et al.* (*17*) have recently shown that in the case of polydisperse system of Gaussian chains the customary interpretation of the intercept and the initial slope of the reciprocal reduced intensity plot to give the weight-average molecular weight ($<M>_w$) and the z-average radius of gyration ($(<r^2>^{1/2}_z$) is valid only when the radius of gyration is less than 1,000 Å, if the angles range above 20°. But, as indicated in Fig. 2, ($P(\theta)$) of our myosin B is fortunately almost constant in spite of wide variation of $<M>_l$. Therefore the polydispersity of the molecular weight has little effect on the shape of the scattering envelope. This indicates that $<M>_l$ is essentially equal to $<M>_w$.

As recorded in Table I, $<M>_l$, that is $<M>_w$, of our myosin B lies in the range of $4.6\text{–}18.2\times10^7$ g., which is higher than that obtained by Morales *et al.* (*6*, *7*). This difference is due to the fact that Morales' preparation contained a larger amount (about 65 per cent of the total) of myosin A than ours.

The scattering envelopes of myosin B curve downward but more gently than in the case of monodisperse rod, as illustrated in Fig. 1, but the polydispersity cannot be one of the main factors determining the shape of the envelope, as discussed above. Hence the reciprocal envelope was compared with the curve calculated for the "worm-like" chain model by Peterlin (*18*) and was found to be closely approximated by the curve of the "worm-like" chain of which the number of the Porod units per molecule is 20–30. The persistence length and the effective diameter of the myosin B molecule are then given as 930–1,080 Å and 6,800–7,900 Å respectively. The fact that the persistence length is longer than the ones of cellulose nitrate (117 Å) and of deoxyribonucleic acid (500 Å) (*19*) suggests the exceptionally low degree of coiling of this protein. Previously Matsumiya and one of the present authors (*20*) found that the rotary diffusion constant (Θ) of the main component is 18–29 sec. and obtained 8,600–10,100 Å for the molecular length in the case of a rigid elongated ellipsoid of the axial ratio of 100. Since a "worm-like" chain is expected to have a smaller value of Θ than that for a ellipsoid of the same length, the Θ previously obtained is in fine correspondence to the length of the "worm-like" chain obtained by the light-scattering method.

As mentioned above, I_θ/c was independent of c in the range of c from 0.05 to 0.5 mg./ml., that is, the value of the second virial coefficient was

essentially zero. Theoretical considerations (*21*) show that the second virial coefficient will be approximately equal to the effective volume of the macromolecules if there is no net attraction between the macromolecules. Therefore, the fact that the second virial coefficient of myosin B is zero indicates that there is fairly strong net attraction between the myosin B molecules, as the very large size of the myosin B molecule would lead to correspondingly large positive value of the second virial coef- ficient.

From the results presented in Fig. 1 and Table I it appears that on the addition of PP $<M>_l$ of myosin B remains constant, but $<r^2>_l^{1/2}$ is greatly increased. Since an unambigous assignment of the type of the average of the molecular weight of myosin B cannot be made in the presence of PP (see however the third section of the next page) because of a somewhat wide variation of $P(\theta)$, it is not absolutely certain but highly probable that the myosin B molecule elongates on the addition of PP without changing the molecular weight. Indeed if we assume according to the suggestion of Gergely and Kohler (*22*) that myosin B consists of 80 per cent of myosin A and 20 per cent of F-actin and it dissociates on the addition of PP completely into myosin A (the molecular weight is about 5×10^5 g.) and F-actin (the molecular weight is about 3×10^6 g.), the number- and the weight-average molecular weights of myosin B at the dissociated state will be respectively about 6×10^5 g. and 10^6 g., both values being extremely lower than $<M>_l$ actually obtained in the presence of PP.

Morales and his associates (*6*, *7*) have already shown the elongation of the myosin B molecule on the addition of PP or ATP and, moreover, they have suggested that ATP elongates myosin B by changing the charge of the protein. But the present authors cannot accept the Morales opinion, partly because Salyrgan, which has no net charge, produces the same effect on the shape of myosin B as PP does, partly because the amount of bound PP necessary to the maximum change of myosin B is, as will be described in the subsequent paper (*8*), one mole per 5.6×10^5 g. protein, much smaller than the value necessary to produce the shape change for muscle contraction by electrostatic repulsion of ATP, predicted by Morales and Botts (*23*), and partly because in the pH range of 6.0–9.5 the size and shape of the protein remain constant, though the charge of the protein changes markedly (according to Dubuisson and Hamoir (*24*) the pH shift from 6.0 to 9.5 produces a change of charge of 35 units per 10^5 g. protein). In a previous paper from this laboratory (*20*), it has been reported that at extremely high gradient (about 10^4 sec.$^{-1}$) the rotary diffusion constant of myosin B is conspicuously increased, in other words, myosin B is fragmented, by ATP-addition. Furthermore, as will be described in the subsequent paper (*8*), it is demonstrated that the units of myosin B of the unit weight of 5.6×10^5 g. behave with regard to the change of light-scattering as if they were almost independent of each other. These observations suggest the looseness of structure of the elongated myosin B molecule. Hence it may be inferred that ATP or PP loosens the protein structure by its binding to the definite site of the protein

(1 mole/5.6×10^5 g. protein) and the protein molecule is elongated by the inherent intramolecular forces, such as the electrostatic repulsion between charged segments of the protein chain.

As recorded in Table III, the content of the light component, which stays in the supernatant under the ultracentrifugal field and has no responce to PP-addition, was increased by the addition of PP from 11 per cent to 30 per cent of the total. Since myosin A has been known to stay in the supernatant under the ultracentrifugal field employed in this work (*13*) and demonstrated by the salting-out analysis (*25*) to be contaminated in our preparation, the light component must be myosin A itself or fragments of myosin B, whose molecular characteristics are very similar to those of myosin A. Hence the above results clearly show that only a small part (about 20 per cent if the dissociable components dissociate to fragments of myosin B or slightly higher than 20 per cent if they dissociate to myosin A and actin; *cf.* (*7*)) of our myosin B dissociates on the addition of PP, confirming directly the ultracentrifugal analysis of von Hippel *et al.* (*7*). This conclusion has been supported from the results of salting-out (*25*) and equilibrium-dialysis (*8*) of the myosin B-PP system.

As previously mentioned, $<M>_l$ remained essentially constant on the addition of PP in spite of the increase in content of the light component. This can be well understood if $<M>_l$ is essentially equal to $<M>_w$ even in the presence of PP and the heavy components which dissociate on PP-addition have relatively low molecular weights. This assumption was supported by the light-scattering measurements on myosin B samples obtained by ultracentrifugation after dilution or storage; their $<M>_l$ and $<r^2>_i^{1/2}$ were smaller than the standard ones and their $<M>_l$ decreased on PP-addition (Table III), thus suggesting the removal by the ultracentrifugation of components of higher molecular weights which do not dissociate but elongate on PP-addition. On the contrary, Gergely (*5*) has interpreted similar results as indicating that components removed by ultracentrifugation after dilution are aggregates which do not react with ATP. However, the present authors cannot accept Gergely's suggestion, because it is apparent from the following three reasons that the components which do not dissociate are really active ones: (i) our myosin B, of which $<M>_l$ remained constant, exhibited drop of I_{90} to 35 per cent of the original on PP-addition, (ii) by ultracentrifugal separation it was shown that the greater part of myosin B did not dissociate on PP-addition and (iii) the pellet which was formed by ultracentrifugation showed ATPase activity identical to the one of the heavy component in the turbid layer.

As illustrated in Fig. 4, the reduced viscosity of myosin B diminished on the addition of PP, though an elongation of macromolecule brings in general an increase in viscosity. This may indicate that the specific viscosity of the dissociable components is greater than the one of the components which elongate on PP-addition and the viscosity of the dissociable components is greatly diminished by the dissociation of the components. If we adopt the

plausible assumption that in the presence of PP the viscosity of the elongated components is much higher than the one of the dissociated ones, the intrinsic viscosity at zero gradient of the elongated myosin B components becomes $8.8/0.7=12.5$ in units of 100 ml./g., as the concentration of the elongated components is about 70 per cent of the total. Applying the Flory-Fox relation (see (*21*)) for the intrinsic viscosity at zero gradient which has been found to apply well to macromolecules of a low degree of coiling (*19*)

$$[\eta]_0=\frac{2.2\cdot 10^{21}<r^2_0>^{3/2}}{P<M>_w}$$

and taking $<M>_w$ as 10^8 g. and P as 2 (*cf.* (*19*)) we obtain 10,500 Å for the root-mean-square end-to-end distance ($<r^2_0>^{1/2}$) of the elongated myosin B. In magnitude this corresponds to the value of the radius of gyration (4,600–6,800 Å) obtained by the light-scattering method.

Thus it follows from various lines of evidence that our myosin B consists of the following three parts: the class of components which take part of 70 per cent of the total and are the "worm-like" chains of the length of 7,500 Å and of the molecular weights of $6.6\text{–}26.0\times 10^7$ g. ($4.6\text{–}18.2\times 10^7$ g./0.7), the class of dis- sociable components which consist of 20 per cent of the total and have much higher viscosity and smaller molecular weights than the main components and the remaining light component which may be myosin A, and that the main components elongate (their radii of gyration become about twice of the original) and the dissociable components dissociate into the light component on the addition of PP.

SUMMARY

1. The angular distribution of the light scattered by myosin B has been determined, and it has been elucidated that the main components of myosin B are the "worm-like" chains having their length of about 7,500 Å.

2. It has been shown by the light-scattering method that on the addition of PP the weight-average molecular weight remains essentially constant but the radius of gyration increases greatly, and that Salyrgan produces the same effect on myosin B as PP does.

3. A considerable decrease of molecular weight on PP-addition has been observed on myosin B ultracentrifuged after dilution or storage.

4. Using the ultracentrifugal separation, it has been shown that the content of myosin A and the like in the preparation increases from 10 to 30 per cent on the addition of PP.

5. From these results it has been concluded that myosin B consists of the following three parts: myosin A (10 per cent), the class of main components (70 per cent) which elongate on PP-addition and the class of dissociable components (20 per cent) which are dissociated by PP.

6. The viscosity of myosin B has been measured as a function of the gradient in the presence and the absence of PP. It has been shown that the viscosity of the dissociable components is much higher than that of the main

components and that the root-mean-square end-to-end distance of the elongated components deduced from the intrinsic viscosity at zero gradient in the presence of PP corresponds well to the radius of gyration by the light-scattering method.

We wish acknowledge a grant from the Ministry of Education of Japan for the purchase of the light-scattering photometer and the ultracentrifuge. We are also indebted to Miss K. Sekiya for her help in determination of the viscosity of myosin B.

REFERENCES

(*1*) Szent-Györgyi, A., Chemistry of Muscular Contraction, Academic Press, New York, 1st ed. (1947)
(*2*) Needham, J., Shen, S. C., Needham, D. M., and Lawrence, A. S. C., *Nature*, **147**, 766 (1941)
(*3*) Dainty, M., Kleinzeller, A., Lawrence, A. S. C., Miall, M., Needham, J., Needham, D. M., and Shen, S. C., *J. Gen. Physiol.*, **27**, 355 (1944)
(*4*) Weber, H. H., and Portzehl, H., *Advances in Protein Chem.*, **7**, 161 (1952)
(*5*) Gergely, J., *J. Biol. Chem.*, **220**, 917 (1956)
(*6*) Blum, J. J., and Morales, M. F., *Arch. Biochem. Biophys.*, **43**, 208 (1953)
(*7*) von Hippel, P. H., Gellert, M. F., and Morales, M. F., Conference on Chemistry of Muscular Contraction, Igaku Shoin Ltd., Tokyo, p. 1 (1957)
(*8*) Tonomura, Y., and Morita, F., *J. Biochem.*, **46**, 1367 (1959)
(*9*) Brice, B. A., Halwer, M., and Speiser, R., *J. Optical Soc. Am.*, **40**, 768 (1950)
(*10*) Oth, A., Oth, J., and Vesreux, V., *J. Polymer Sci.*, **10**, 551 (1953)
(*11*) Brice, B. A. and Halwer, M., *J. Optical Soc. Am.*, **41**, 1033 (1951)
(*12*) Zimm, B. H., *J. Chem. Phys.*, **16**, 1099 (1948)
(*13*) Weber, A., *Biochim. et Biophys. Acta*, **19**, 345 (1956)
(*14*) Claesson, S., and Lohmander, U., *Makromol. Chem.*, **18–19** (Staudinger-Festband), 310 (1956)
(*15*) Krieger, T. M., and Maron, S. H., *J. Appl. Phys.*, **23**, 147 (1952)
(*16*) Bailey, K., *Biochem. J.*, **43**, 271 (1948)
(*17*) Benoit, H., Holtzer, A. M., and Doty, P., *J. Phys. Chem.*, **58**, 635 (1954)
(*18*) Peterlin, A., *J. Polymer Sci.*, **10**, 425 (1953)
(*19*) Doty, P., *J. Cell. Comp. Physiol.*, **49**, Suppl. 1, 27 (1957)
(*20*) Tonomura, Y., and Matsumiya, H., *Biochim. et Biophys. Acta*, **27**, 311 (1958)
(*21*) Flory, P. J., Principles of Polymer Chemistry, Cornell Univ. Press, Ithaca, (1953)
(*22*) Gergely, J., and Kohler, H., Conference on Chemistry of Muscular Contraction, Igaku Shoin Ltd., Tokyo, p. 14 (1957)
(*23*) Morales, M. F., and Botts, J., *Arch. Biochem. Biophys.*, **37**, 283 (1952)
(*24*) Dubuisson, M., and Hamoir, G., *Arch. Intern. Physiol.*, **53**, 308 (1943)
(*25*) Sasaki, A. T., *J. Research Inst. Catalysis* (*Hokkaido Univ.*), **6**, 221 (1958)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

THE BINDING OF PYROPHOSPHATE TO MYOSIN A AND MYOSIN B

By YUJI TONOMURA and FUMI MORITA

(From the Research Institute for Catalysis and the Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo)

(Received for publication, March 27, 1959)

For elucidating the mechanism of the reaction between myosin A or myosin B and adenosine triphosphate (ATP) or inorganic pyrophosphate (PP) as well as for getting insight into the physico-chemical mechanism of muscle contraction, it is important to determine the strength of the bond between myosin A or B and these phosphates and the maximum number of sites of these proteins available to ATP or PP. The number of sites and the strength of binding of ATP to myosin B were first studied by Mommaerts (*1*, *2*) by observing the change of viscosity of myosin B solution by ATP-addition, and later they were estimated in our laboratory (*3*, *4*) from the change of light-scattering with a rapid method. Recently, Mommaerts *et al.* (*5*, *6*) have estimated these two characteristic values of the myosin B-ATP system from the light-scattering change by ATP in the presence of pyruvic kinase and phosphoenolpyruvate. However, these studies have been able to give only indirect informations on the binding of ATP to myosin B. Gergely and Kohler (*7*) have determined the number of sites of myosin A available to PP, but measured it only at a fixed concentration of PP. No systematic and direct study on this important problem has yet been reported.

The present paper is concerned with the determinations of the number of the available sites and the strength of binding of PP to myosin A and myosin B by the equilibrium-dialysis method and the comparison of the extent of binding of PP with the degree of the change of light-scattering of myosin B. It is concluded that in the case of myosin A two binding sites are available per molecule, but in the case of myosin B only one of these two sites is available to PP-binding and that the intensity of light scattered by myosin B is decreased by an almost constant value every time one PP molecule combines to one binding site of myosin B.

METHODS

Materials—Myosin B was prepared as described previously (the "standard" sample in the preceding paper (*8*)) and myosin A was prepared as described by Perry (*9*).

Inorganic pyrophosphate labelled with P^{32} (PP*) was prepared as follows: the solution, containing carrier free P^{32} and 0.1 *M* Na_2PO_4, was adjusted to pH 9.0 by NaOH, evaporated to dryness, and then heated to 230° for 24 hours. After cooling PP* was twice recrystallized.

Procedures—The equilibrium-dialysis was carried out by the method of Klotz (*10*). Cellophane bags were prepared from Visking Nojax sausage casing, 23/32 inch in diameter and filled with 10 ml. of the solution containing the protein (about 6 mg./ml.), 0.6 or 0.04 *M* KCl, the measured amount of $MgCl_2$ or $CaCl_2$ and 0.01 *M*-Tris-maleate buffer adjusted to pH 7.5. Ten ml. of 0.6 or 0.04 *M* KCl solution containing the measured amount of PP* and $MgCl_2$ or $CaCl_2$ was used in the tube outside the bag. The tubes were shaken in a bath at 5° for 48-96 hours. To eliminate the error due to the phosphate contamination in the protein preparations, the concentration of PP* in the outer solution was determined by measuring the radioactivity with an aid of a Geiger counter (Kaken, Model 16) after an attainment of equilibrium. For each PP concentration a control tube was also prepared which differed from the primary tube only in that the former contained the solvent rather than the protein solution inside the bag. Correction for the concentration of free PP* was made by measuring concentration of orthophosphate produced during the run by the breakdown of PP* that occurred naturally or owing to the pyrophosphatase contamination in the protein preparations. Because of rather small amount of binding, the extent of binding of PP (ν) could not be measured accurately when the concentration of PP was high; for example at 10^{-4} *M* PP the error in ν was about ±5–10 per cent. Since the relation between concentration of free PP ([PP]) and ν was not usually represented by a dissociation curve of the first order, $\nu=1/(1+K/[PP])$ (*11*), the maximum number of sites was determined directly by measuring the constant ν independent of [PP] at concentration as high as possible. The extrapolation method was used only if unavoidable.

Intensity of scattered light at the angle of 90° from incident (I_{90}) was measured by an apparatus described previously (*12*), current from a photomultiplier being measured by a micro-microammeter (Ohkura, Model AM102). The measurements were made in 0.6 *M* KCl solution at pH 7.5 and 5°, protein concentration being 0.5–1 mg./ml. A Brice-Phoenix photometer (*13*) was employed only when the angular distribution was measured.

The content of protein was calculated by multiplying a factor of 6 the nitrogen content determined by the micro-Kjeldahl method (*14*).

RESULTS

Change of Light-Scattering—In the preceding paper (*8*) it has been shown that our myosin B preparation consists of 10 per cent of myosin A, 70 per cent of the main components and 20 per cent of the dissociable ones and that by the addition of PP the main components are elongated with conatant molecular weights and the dissociable ones are dissociated into myosin A and the like. Since the myosin B-PP system is complicated and heterogeneous in this manner, the measurement of the angular dependence of the relation between the degree of change of light-scattering (Δ) and [PP] might be useful to elucidate the mechanism. Here Δ is defined by

$$\Delta=(I_0-I_s)/(I_0-I_\infty)$$

where I_0 and I_s are the intensities of scattered light before and after the addition of some amount of PP respectively and I_∞ after the addition of sufficient amount of PP. However as recorded in Table I, Δ was almost independent of the angle. Taking advantage of this fact, the intensity of scattered light was measured exclusively at 90° in what follows.

Since the concentration of protein and the time of incubation employed

in the light-scattering method were much different from these in the equilibrium-dialysis, the dependence of the relation $\Delta-[PP]$ on these two factors was examined. It was observed that the relation $\Delta-[PP]$ was independent of

TABLE I

The Angular Dependence of Degree of Change of Scattered Light (Δ) *in the Presence of PP*

Intensity of scattered light was measured in 0.6 M KCl and 10^{-3} M $MgCl_2$ and at pH 8.0 and 26° in the presence and the absence of 1.2×10^{-6} M PP. The concentration of protein (sample No. VIII 11) was 0.01 mg./ml.

Angle	45°	53°	66°	90°	114°	127°
Δ (*per cent*)	37.9	35.0	37.5	35.7	43.8	37.5

the concentration of protein in the range from 0.2 to 6 mg./ml. where the both measurements were made, but Δ increased with decrease of the protein concentration when the protein concentration was lower than 0.1 mg./ml., and I_{90} of the myosin B-PP system changed little during 80 hours at 5°.

The effects of various divalent cations on the change by PP of light scattered by myosin B were measured in advance. In general smaller cations (smaller than 0.8 Å in ionic radius) were more effective than larger ones (larger than 0.99 Å in ionic radius). For example, in the presence of $10^{-5.55}$ M PP and 10^{-3} M of $MnCl_2$, $CoCl_2$ or $MgCl_2$, Δ of sample No. VIII 12 was found to be about 47 per cent, irrespective of the kind of cation, while in

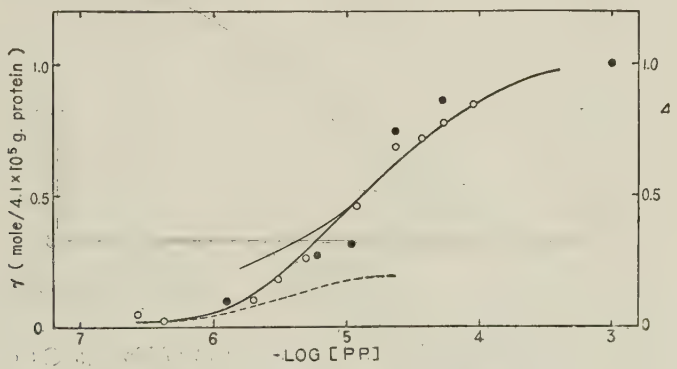


FIG. 1. The binding of PP to myosin B (sample No. VIII 13) as a function of concentration of free PP.

0.6 M KCl, 3×10^{-4} M $MgCl_2$, pH 7.5, 5°. Extent of binding, ○; degree of change in light-scattering, ●. The fine solid line represents the theoretical one: $\nu=\dfrac{1}{1+10^{-4.7}/[PP]}$. The dotted line illustrates the binding curve due to minor component(s).

the presence of 10^{-3} *M* $CaCl_2$, $SrCl_2$ or $BaCl_2$ the intensity of light-scattering decreased only slightly. In the experiments reported here calcium ion and, more principally, magnesium ion were used as representatives of these two types of modifiers, because these cations are most interesting physiologically.

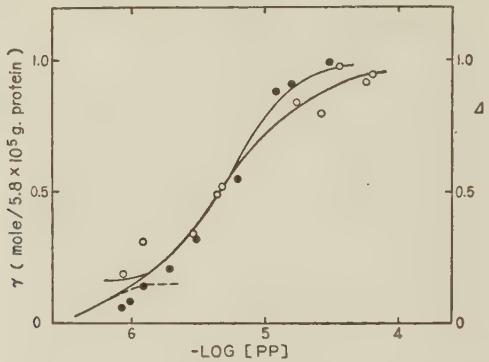


FIG. 2. The binding of PP to myosin B (sample No. VIII 15) as a function of concentration of free PP.

0.6 *M* KCl, 3×10^{-4} *M* $MgCl_2$, pH 7.5, 5°. Extent of binding, ○; degree of change in light-scattering, ●. The fine solid line represents the theoretical one: $\nu=\frac{1}{1+(10^{-5.25}/[PP])^2}$. The dotted line illustrates the binding curve due to minor component(s).

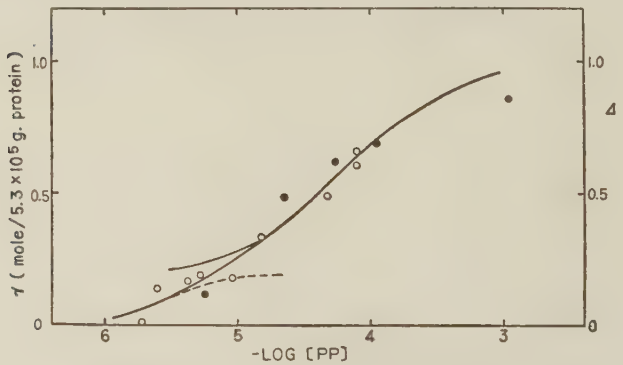


FIG. 3. The binding of PP to myosin B (sample No. VIII 17) as a function of concentration of free PP.

0.6 *M* KCl, 10^{-4} *M* $CaCl_2$, pH 7.5, 5°. Extent of binding, ○; degree of change in light-scattering, ●. The fine solid line represents the theoretical one: $\nu=\frac{1}{1+10^{-4.25}/[PP]}$. The dotted line illustrates the binding curve due to minor component(s).

In Figs. 1, 2 and 3 are plotted the degree of light-scattering change as a function of [PP] and in Table II are summarized the results. As already reported

by us (*11*) the relation $\Delta - [PP]$ fluctuated greatly from one sample to another.

TABLE II

The Amount of Unit Weight (Amount of Protein per One Mole of Binding Site) of Myosin B and the Strength of Binding of PP to Myosin B

Extent of binding of PP was measured in 0.6 *M* KCl and at pH 7.5 and at 5°

Myosin B No.	Divalent cation	Unit weight × 10^{-5} g.	Dissociation constant				Minor comp. (*per cent*)
			Chemical		Optical		
			order	*pK*	order	*pK*	
VIII 9	3×10^{-4} *M* $MgCl_2$	5.8	1	5.1	1	5.1	10*
VIII 13	3 ,, $MgCl_2$	4.4	1	4.7	1	4.8	10
VIII 14	3 ,, $MgCl_2$	5.0	2	4.4	2	—	10
VIII 15	3 ,, $MgCl_2$	6.5	2	5.25	2	5.25	7.5
VIII 16	3 ,, $MgCl_2$	5.0	2	5.5	2	5.25	15
VIII 17	1 ,, $CaCl_2$	5.9	1	4.25	1	4.3	10
VIII 18	1 ,, $CaCl_2$	5.8	1	4.15	1	4.15	10*

* Value estimated from data of ultracentrifugal separation.

The number of sites of myosin A or B available to ATP cannot be measured by the equilibrium-dialysis method, as ATP is hydrolyzed by myosin. Even the measurements with a rapid light-scattering method previously used by us (*3*) might not be satisfactory because of hydrolysis of ATP, as commented by Nanninga and Mommaerts (*6*). Then, the minimum quantity of ATP necessary to cause the maximum change in light-scattering was measured in the presence of various concentrations of $MgCl_2$, which inhibits adenosine triphosphatase and intensifies the strength of the binding of ATP. Since high concentration of magnesium ion forms precipitates with ATP, the limiting value at sufficiently high concentration of magnesium ion was inferred by extrapolation and estimated to be one mole ATP per 4–6× 10^5 g. protein (Fig. 4).

Equilibrium-Dialysis—A typical example of binding of PP to myosin A (M) in the presence of $MgCl_2$ is illustrated in Fig. 5 and the results are summarized in Table III. It is apparent from these results that one mole of PP can combine to $2.3(\pm0.25)\times10^5$ g. of myosin A. This means, as will be mentioned under "Discussion", that the number of the binding sites per myosin A molecule is two. If we assume that these two sites are identical with each other and the binding of PP follows the mass action law, the relation between

the extent of binding (ν) per myosin A molecule and [PP] is given by

$$\nu = \frac{\frac{[PP]}{K_1}\left\{1+\frac{2[PP]}{K_2}\right\}}{1+\frac{[PP]}{K_1}\left\{1+\frac{[PP]}{K_2}\right\}},$$

where K_1 and K_2 represent the dissociation constants of the reactions $M+PP \rightleftarrows MPP$ and $MPP+PP \rightleftarrows (PP)_2$ respectively. Although the accuracy of ν was

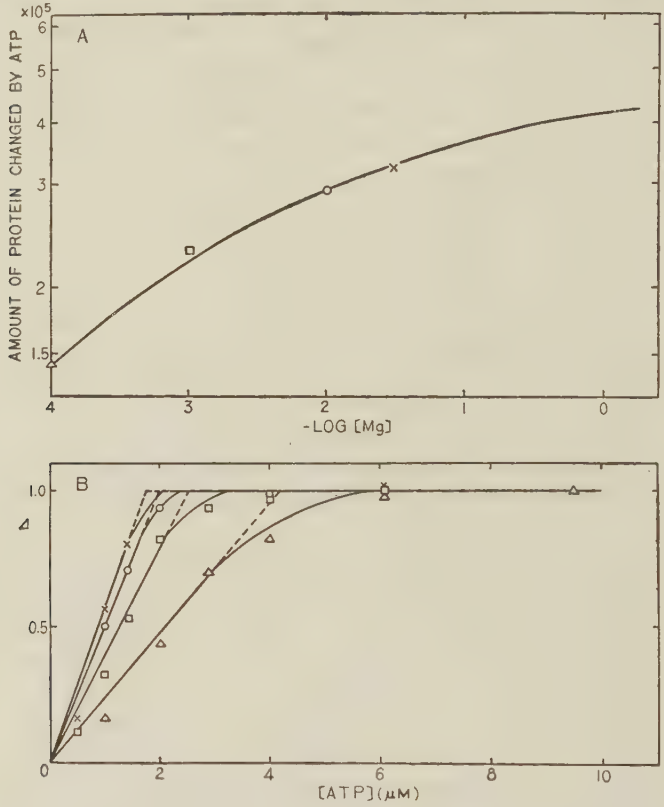


FIG. 4. The minimum amount of ATP necessary to maximum change in light-scattering.

Myosin B No. VIII 19. 0.6 mg./ml. 0.6 M KCl, pH 6.9, 15°. $MgCl_2$ 10^{-4} M, Δ; 10^{-3} M, □; 10^{-2} M, ○; 3×10^{-2} M, ×.

not so high to evaluate K_1 and K_2 accurately, the ν calculated by K_1 and K_2 values listed in Table III showed satisfactory agreement with the experimental one. The K_1 and K_2 values were about $10^{-6.2}$ and $10^{-5.3}$ M respectively in the presence of 0.6 M KCl and 3×10^{-4} M $MgCl_2$. These dissociation constants are much larger than Gergelys' so-called "intrinsic" one (approximately 10^{-8} M at an almost identical condition) estimated by them (7) by an indirect method. It is also interesting to note that both pK_1 and $\Delta pK(=pK_1-pK_2)$

increase with decrease of ionic strength of medium (Table III).

In the presence of $CaCl_2$, the saturated value of ν could not be obtained, as the binding occurred appreciably at rather high concentration of PP. But at [PP] of $10^{-4.4}$ M, ν was found to exceed one mole per 3.4×10^5 g. protein.

The extent of binding of PP to myosin B and the degree of change in light-scattering are illustrated in Figs. 1, 2 and 3 as a function of [PP]. It

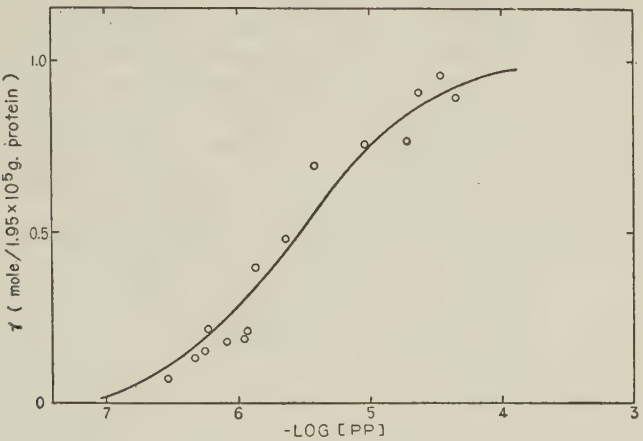


FIG. 5. The binding of PP to myosin A (No. VIII 2) as a function of concentration of free PP.

0.6 M KCl, 3×10^{-4} M $MgCl_2$, pH 7.5, 5°. The line represents the theoretical one: $\nu=\dfrac{10^{5.9}\,[PP]\,(1+2\times10^{5.2}\,[PP])}{1+10^{5.9}\,[PP]\,(1+10^{5.2}\,[PP])}$.

TABLE III

The Amount of Unit Weight (Amount of Protein per One Mole of Binding Site) of Myosin A and the Strength of Binding of PP to Myosin A

Extent of binding of PP was measured at pH 7.5 and at 5°.

Myosin A No.	Ionic medium	Unit weight × 10^{-5} g.	Dissociation constants	
			pK_1	pK_2
VIII 1	0.6 M KCl 3×10^{-4} M $MgCl_2$	2.45	6.3	5.4
VIII 2	0.6 M KCl 3 ,, $MgCl_2$	1.95	5.9	5.2
VIII 3	0.04 M KCl 3 ,, $MgCl_2$	2.5	6.8	4.7
VIII 4	0.6 M KCl 1 ,, $CaCl_2$	< 3.4	(5.7)	—

was our usual experience that the relation ν – [PP] did not follow exactly a dissociation curve of the first order, $\nu=1/(1+K/[PP])$, or the second order, $\nu=1/\{1+(K/[PP])^2\}$, that is, over the range of low [PP] an obvious deviation

was usually observed. Attributing this deviation of PP-binding to contamination of minor component(s) in our myosin B preparation, pK and the order of binding of PP to the major component could be determined from the binding curve except the portion due to minor contaminant(s). The pK and the order calculated in this manner are listed in Table II for seven myosin B preparations in the presence of $MgCl_2$ or $CaCl_2$. The pK and the order fluctuated from one preparation to another, as in the change of light-scattering (*11*). However, the pK of the major component of myosin B was mostly smaller than the one of myosin A. But the most remarkable fact is that the pK and the order agree almost with those obtained by the light-scattering measurement. In the case of binding of the first order the agreement between the relation $\nu - [PP]$ of the major component and the one $\Delta - [PP]$ was rather satisfactory, but in the case of the second order the agreement was not so good, especially over the range of high [PP] (Fig. 2).

As will be mentioned under "Discussion", the minor component, to which PP binds within the range of low [PP], has been identified with myosin A contaminated in our preparation. Taking the amount of sites of myosin A as one mole per 2.3×10^5 g. protein, the content of the minor component could be roughly estimated to be 7.5–15 per cent of the total, as listed in Table II. This value agrees well with the one determined by the ultracentrifugal separation (*8*). Hence, when content of the minor component could not be determined from the binding curve, it was *a priori* assumed to be 10 per cent of the total. The unit weight of myosin B, *i.e.* the amount of protein per mole of the binding site, thus calculated are listed in Table II. These are constant, within experimental error, regardless of the order of binding and kind of divalent cation. The mean unit weight 5.6 $(\pm0.67)\times10^5$ g., are larger than twice of that of myosin A.

DISCUSSION

As described in "Results", 2.3 $(\pm0.25)\times10^5$ g. of myosin A can bind one mole of PP. Although the value of 8.4×10^5 g. (*15*, *16*) was often cited as the standard figure of the molecular weight of myosin A, Mommaerts and Aldrich (*17*) and von Hippel *et al.* (*18*) have recently obtained a molecular weight of 4.2×10^5 g. by the Archibald approach to sedimentation equilibrium method, and Gergely and Kohler (*7*) and also Nihei and Tonomura* have obtained 5.0×10^5 g. by the light-scattering method. Therefore our result on the binding demonstrates that the number of binding sites available to PP is two per myosin A molecule. Then the question arises as to whether these two sites of myosin A are identical with respect to PP-binding or not. If the sites are different from and independent of each other, the relation $\nu - [PP]$ is given by

$$\nu = \frac{1}{1+\frac{K_1}{[PP]}} + \frac{1}{1+\frac{K_2}{[PP]}},$$

* unpublished experiments.

where K_1 and K_2 are the dissociation constants of the binding of PP to the first and the second sites. We could not choose experimentally between the above equation and the one adopted in "Results" which is derived under the assumption of the identical sites owing to small difference of the equations. But the assumption of the identical sites seems to be preferable from the following reasons: (i) this assumption is compatible with the Laki model of myosin A (*19*), (ii) it gives a reasonable explanation of the result that decrease in ionic strength brings increase of ΔpK, the standard free energy of the electrostatic repulsion between PP molecules absorbed to the identical sites being proportional to $(\Delta pK - \log^4)$, and (iii) the observation made by us (*20*), that adenosine triphosphatase activity of myosin B is almost half as high as the one of myosin A contained in the same preparation, supports this assumption, since the amount of the binding site of myosin A is about twice as high as the one of myosin B and there are good evidences (4, *21*, *22*) for believing that the active site of adenosine triphosphatase is nothing but the binding site for the size and shape change of myosin B by ATP or PP.

In the case of myosin B, existence of two components, the major and the minor, was deduced from heterogeneity of the binding curve. The major component must be myosin B itself (referred as the "heavy" components in the preceding paper), because the intensity of light-scattering drops as PP binds to this component (see Table II and Figs. 1–3). On the other hand, the minor component is in all possibilities myosin A contaminated in our preparation from the following three reasons: (i) The combination of PP to this component seems to bring no change in intensity of scattered light. In fact we have tried carefully several times to find out heterogeneity of Δ within the range of low [PP], but the heterogeneity has not been observed. (ii) The minor component binds PP within the range of lower [PP] than the major component does. (ii) Its content agrees well with the content of myosin A estimated by the ultracentrifugal separation (*8*).

Following these identifications of the components, the unit weight of the heavy components of myosin B was calculated to be 5.6 $(\pm 0.67) \times 10^5$ g., as recorded in Table II. (If the minor component is other protein(s) than myosin A and its unit weight is smaller than the one of myosin B, the unit weight of the heavy components must be slightly larger than the value listed in Table II, taking the content of myosin A as 10 per cent. On the other hand, if the minor component is other protein(s) than myosin A and its unit weight is higher than myosin B, it will be deduced that our myosin B preparation is contaminated with more than 10 per cent of unknown protein(s) other than myosin A. It is highly unprobable from the salting-out analysis of our preparation (*23*)). If myosin B dissociates completely into myosin A and F-action on PP-addition, the unit weight of myosin B must be smaller than 3.3×10^5 g., because it is a well-established fact that more than 70 per cent of myosin B consists of myosin A (*24*, *25*). Therefore the above result is well compatible with the conclusion of the preceding paper that the majority of myosin B does not dissociate but elongates on the addition of PP.

Since it has already been shown by Yagi (*26*) that the minimum amount of ATP necessary to the maximum change in light-scattering is independent of the content of actin in myosin B preparation, it is highly probable that PP also binds to the myosin A molecules of the heavy components. Then the results reported here lead us to the following two *a priori* possible interpretations. First, if we assume that two molecules of PP can bind to one molecule of myosin A which constitutes the heavy components the ratio of contents of myosin A and actin in the heavy components becomes 2.3 : (5.6−2.3)=about 2 : 3. This is inconsistent with the well-established fact that the main constituent of myosin B is myosin A. Second, if only one of the two sites is available to PP in myosin A of the class of main components (78 per cent of the heavy components) which elongate, the other being occupied by actin, while the both sites are available in the class of dissociable components (22 per cent) which dissociate into myosin A and the like on PP-addition (*8*), then the ratio of myosin A to actin in the heavy components becomes 1 : $\{5.6\times(0.78/4.6+0.22/2.3)-1\}=2.1:1$. As the ratio is not known with certainty but it may be 2.5 : 1 (*24*, *25*), the second case is legitimate, though, of course, the ratio calculated cannot be exact owing to low accuracy of the unit weights.

As described above, the extent of the binding of PP to the heavy component agrees, at least as a first approximation, with the degree of change in light-scattering, thus proving the assumption tacitly adopted by many workers (*4*–*6*, *22*, *27*) in this field, that the degree of the light-scattering change of myosin B is equal to the degree of the size and shape change of unit of myosin B. This fact indicates that the units of myosin B behave with regard to the change of light-scattering as if they were almost independent of each other and the intensity of light scattered by myosin B is decreased by an almost constant value every time one PP molecule combines with one myosin B unit. The molecular mechanism underlying this simple relation is not yet clear. This point will be a subject of a future research.

Whether ATP and PP occupy the same site of myosin A and B or not is unsettled, since the binding of ATP cannot be measured directly by the equilibrium-dialysis method. However, the above conclusions deduced on PP-binding may be applicable also to ATP-binding, because ATP produces the same size and shape change in myosin B as PP does (*24*, *25*) and the unit weight of myosin B with respect to ATP-binding measured by the light-scattering method agrees well, as above mentioned, with the one of PP-binding.

SUMMARY

1. The minimum quantities of ATP necessary to cause the maximum change in light scattered by myosin B have been measured in the presence of various concentrations of $MgCl_2$. By extrapolation to sufficiently high concentration of $MgCl_2$, it has been found that one mole of ATP is able to produce the maximum change in the physical state of $4–6\times10^5$ g. of myosin B.

2. The bindings of PP to myosin A and myosin B have been investigated

by the equilibrium-dialysis method. It is confirmed that myosin A can bind one mole of PP per 2.3×10^5 g. protein. This means that there are two binding sites per myosin A molecule. On the other hand, the amount of binding site of the heavy component of myosin B is one mole per 5.6×10^5 g. protein.

3. From these results it is concluded that one of the two sites of the myosin A molecule which constitutes the main component of myosin B is occupied by actin and PP can bind with the other.

4. The extent of the binding of PP to the heavy component agrees, at least as a first approximation, with the degree of change in light-scattering of myosin B, thus indicating that the units of myosin B behave with regard to the change of light-scattering as if they were nearly independent of each other.

This study has been aided in part by a grant-in-aid for Fundamental Scientific Research of the Ministry of Education of Japan given to the Research Group on the "Mechanism of Enzyme Action."

REFERENCES

(*1*) Mommaerts, W. F. H. M., *Studies Inst. Med. Chem. Szeged*, **1**, 37 (1942)
(*2*) Mommaerts, W. F. H. M., *J. Gen. Physiol.*, **31**, 361 (1948)
(*3*) Tonomura, Y., Watanabe, S., and Yagi, K., *J. Biochem.*, **40**, 27 (1953)
(*4*) Tonomura, Y., *J. Research Inst. Catalysis (Hokkaido Univ.)*, **4**, 259 (1956)
(*5*) Mommaerts, W. F. H. M., and Hanson, J., *J. Gen. Physiol.*, **39**, 831 (1956)
(*6*) Nanninga, L. B., and Mommaerts, W. F. H. M., *Proc. Natl. Acad. Sci.*, **43**, 540 (1957)
(*7*) Gergely, J., and Kohler, H., Conference on Chemistry of Muscular Contraction, Igaku Shoin Ltd., Tokyo, p. 14 (1957)
(*8*) Nihei, T., and Tonomura, Y., *J. Biochem.*, **46**, 1355 (1959)
(*9*) Perry, S. V., Methods in Enzymology, Academic Press, New York, Vol. **2**, p. 582 (1955)
(*10*) Hughes, T. R., and Klotz, I. M., Method of Biochemical Analysis Interscience Pub., New York, Vol. **3**, p. 265 (1953)
(*11*) Tonomura, Y., Morita, F., and Yagi, K., *J. Phys. Chem.*, **61**, 605 (1957)
(*12*) Matsumiya, H., Morita, F., Kitagawa, S., Yagi, K., and Tonomura, Y., *J. Biochem.*, **44**, 347 (1957)
(*13*) Brice, B. A., Halwer, M., and Speiser, R., *J. Optical Soc. Am.*, **40**, 768 (1950)
(*14*) Bailey, K., *Biochem. J.*, **43**, 271 (1948)
(*15*) Portzehl, H., Schramm, G., and Weber, H. H., *Z. Naturforsch.*, **5b**, 61 (1950)
(*16*) Portzehl, H., *Z. Naturforsch*, **5b**, 75 (1950)
(*17*) Mommaerts, W. F. H. M., and Aldrich, B. B., *Biochim. et Biophys. Acta*, **28**, 627 (1958)
(*18*) von Hippel, P. H., Schachman, H. K., Appel, P., and Morales, M. F., *Biochim. et Biophys. Acta*, **28**, 504 (1958)
(*19*) Laki, K., *J. Cell. Comp. Physiol.*, **49**, Suppl. 1, 249 (1957)
(*20*) Yasui, T., Hashimoto, Y., and Tonomura, Y., *Arch. Biochem. Biophys.*, in press
(*21*) Bailey, K., and Perry, S. V., *Biochim. et Biophys. Acta*, **1**, 506 (1947)
(*22*) Tonomura, Y., Matsumiya, H., and Kitagawa, S., *Biochim. et Biophys. Acta*, **24**, 568 (1957)

(23) Sasaki, A. T., *J. Research Inst. Catalysis* (*Hokkaido Univ.*), **6**, 221 (1958)
(24) Szent-Györgyi, A., Chemistry of Muscular Contraction, Academic Press, New York, 1st ed. (1947)
(25) Weber, H. H., and Portzehl, H., *Advances in Protein Chem.*, **7**, 161 (1952)
(26) Yagi, K., *J. Biochem.*, **44**, 337 (1957)
(27) Blum, J. J., *Arch. Biochem. Biophys.*, **55**, 568 (1957)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

STUDIES ON HOMOGENTISICASE*

I. PURIFICATION AND THE ROLE OF FERROUS ION IN THE ENZYMATIC ACTION

By KEIKO TOKUYAMA

(*From the Institute for Protein Research, Osaka University, Osaka*)

(Received for publication, March 30, 1959)

The first study of homogentisicase which catalyses the oxidation of homogentisic acid was made by Suda and Takeda (*1*). Using partially purified enzyme preparation isolated from rabbit liver, these authors reported that ferrous ion was required to activate the enzyme, that other metal ions were ineffective, and that the enzyme was strongly inhibited by α,α'-dipyridyl but not by cyanide. These findings were subsequently confirmed in part and further extended by Crandall (*2*, *5*), Schepartz (*3*), Knox *et al.* (*4*, *6*) and Suda (*7*). The first three of the investigators, however, reported that the enzymatic reaction was inhibited by cyanide and azide.

The present study is concerned with purification and properties of the enzyme, which was isolated from acetone-dried beef liver. Using labeled iron salts, it was demonstrated that during the reaction the ferrous ion attached to it became mobilized and exchanged with exogenous Fe^{59}. The mode of action of the enzyme will also be discussed in the present paper, with special reference made to the role of the ferrous ion.

METHODS

The activity of homogentisicase was measured at 30° by the manometric method. The standard reaction mixture consisted of an appropriate volume of the enzyme solution, 0.4 ml. of 0.2 *M* phosphate buffer, pH 7.2, 0.2 ml. of 0.01 *M* $FeSO_4$, 0.2 ml. of 0.02 *M* homogentisic acid and a sufficient amount of distilled water to make the final volume 2.0 ml. When the specific activity of the enzyme was measured, 0.2 ml. of 0.01 *M* glutathione or ascorbic acid was added, but the final volume was always kept at 2.0 ml. The specific activity of the enzyme was expressed in terms of oxygen taken up in μl. per hour per mg. of protein as measured at 30°.

The amount of protein was determined from the value of optical density at 660 mμ by the use of the Folin-Ciocalteu phenol reagent (*8*); that of ferrous ion was estimated as the value of optical density at 500 mμ by the *o*-phenanthroline method (*9*); and that of the ferric ion was estimated colorimetrically by the thiocyanate method.

* This study was supported by a Grant for Scientific Research from Ministry of Education. A part of this work was reported at the 14th International Congress of Biochemistry held in Vienna, September, 1958.

The Fe^{59} exchange experiments were conducted in an apparatus which was previously employed for the study of pyrocatechase (*10*). The method used was a modification of the one described by Dawson *et al.* (*11*). The apparatus was so designed that, after the reaction, the reaction mixture was passed through a column containing the cation exchange resin, Dowex 50×8, to remove the free iron. The enzyme-bound iron appeared in the effluents, together with the protein moiety. The radioactivity of the effluents was measured by the thin-window type Geiger-Müller counting system. The Fe^{59} preparation used was a mixture of ferrous and ferric chlorides.

Homogentisic acid was isolated from alcaptonic urine according to the procedure developed by Kiyokawa *et al.* (*12*) with slight modification. The melting point of the recrystallized preparation was 146°.

Maleylacetoacetic acid was prepared by deproteinizing the reaction mixture with trichloracetic acid after the completion of the reaction with the isomerase-free Fe^{++}-containing enzyme.

RESULTS

Purification of Homogentisicase—Acetone-dried powder of beef liver was used as the starting material to prepare homogentisicase. All the purification procedures were carried out at or below 4°. Ten grams of acetone-dried powder were extracted for 16 hours in a refrigerator with 200 ml. of distilled water, adjusted to pH 9.0 with 5 per cent aqueous ammonia. After filtrating the extract with the aid of 5 g. of celite, 1.2 g. solid sodium chloride was added to 200 ml. of filtrate and then 100 ml. of chilled acetone was slowly added −5° with vigorous stirring. The precipitate collected by centrifugation (3,600× *g*, 10 minutes) at about −5°, was dissolved in 40 ml. of distilled water which had been brought to pH 8.0 with aquous ammonia. Solid ammonium sulfate was added to the above solution until its saturation became 0.15. After the precipitate was removed by centrifugation (5,100×*g*, 10 minutes), the supernatant fluid was fractionated with ammonium sulfate at pH 7.4. The precipitate produced between 0.45 and 0.80 saturation was collected by centrifugation (8,100×*g*, 15 minutes) and dissolved in 20 ml. of distilled water which was made slightly alkaline (pH 7.6) with aqueous ammonia. Four milliliters of calcium phosphate gel suspension* (wet weight 0.2 g./ml.) were added to the solution, and the mixture was allowed to stand for 10 minutes. The mixture was then centrifuged (2,700×*g*, 3 minutes) and the precipitate was discarded. The supernatant fraction was free of the cis-trans isomerase**, which catalyses the isomerization of maleylacetoacetic acid to fumarylacetoacetic acid. The supernatant was then dialysed for 2 hours against the chilled phosphate buffer (0.01 *M*) of pH 7.0 or acetate buffer (0.01 *M*) of pH 5.0 with continuous stirring. The enzyme solution dialyzed at pH 7.0 was called the "Fe^{++}-containing enzyme" (or simply "Fe^{++}-enzyme"), since the enzyme in

* The calcium phosphate gel was prepared by the method of Tsuchihashi (*13*).

** According to Knox *et al.* (*14*) the assay of *cis-trans* isomerase was made in the presence of glutathione which participates as a coenzyme. No isomerisation of maleylacetoacetic acid could be observed.

this preparation still retained its protein-bound ferrous ion*. The dialyzed solution obtained at pH 5.0 was centrifuged (9,600×*g*, 5 minutes) to remove insoluble materials and the supernatant was adjusted to pH 6.8 with aqueous ammonia. This preparation was called the "Fe^{++}-free enzyme", since in this preparation the essential ferrous ion was removed from the enzyme protein during dialysis*. The preparation of Fe^{++}-enzyme as well as Fe^{++}-free enzyme was transparent. The specific activity and the yield at each step of purification are shown in Table I. As can be seen, the purified Fe^{++}-free enzyme preparation obtained at the final step represented a 50-fold increase in the specific activity.

TABLE I

Specific Activities and Yields of Preparations at Each Step of Purification

Activity was measured at 30° by manometric method. Reaction mixture consisted of an appropriate volume of the enzyme solution, 0.4 ml. of 2×10^{-1} *M* phosphate buffer, pH 7.2 ml. of 1×10^{-2} *M* $FeSO_4$, 0.2 ml. of 2×10^{-2} *M* homogentisic acid, 0.2 ml of 1×10^{-2} *M* glutathione and sufficient amount of distilled water to make the final volume 2 ml.

Stage of Purification	Protein content (*mg./ml.*)	Specific activity (*μl. O_2/hour/mg. protein*)	Total volume (*ml.*)	Yield (%)
Initial material (5 g. of acetone-dried powder extracted with 100 ml. dist. water)	16	20	100	100
Filtrate	13	25	95	96
Sample obtained after acetone fractionation (0.33 saturation)	8	62	20	31
Sample obtained at 0.8 saturation of ammonium sulfate	2.6	250	10	20
Supernatant obtained after treatment with calcium phosphate gel	1.6	390	10	20
Sample obtained by dialysis at pH 5.0	0.75	980	14	35

Stability of Purified Enzymes—Although any measurable decrease in activity was not observed during the experiments, both preparations were susceptible to aging on storage. The activity of the enzyme, when stored overnight at pH 6.0–7.6 at 0°, was reduced to 40–70 per cent of its original value. Such

* Data will be given in a later section.

inactivation was also observed even when they were stored in dry ice. Neither ferrous salts nor proteins such as bovine serum albumin showed any protective effects against inactivation. It was, however, found that glutathione or ascorbic acid (10^{-3} *M*) was remarkably effective in preventing inactivation of the enzyme during storage at 0°; no loss or but a slight loss of activity was detected after 24 hours of storage.

The preparations were also found to be rapidly inactivated when they were placed in the media of higher acidity than pH 4.0. They were, however, proved to be stable over a wide range of pH for short incubation periods. After the enzyme solution buffered at various pH's was kept to stand at 28° for 1.5 hours, the pH of the solution was brought to 6.3 and ferrous ion (10^{-3} *M*) was added. After 10 minutes of incubation, the reaction was initiated by the addition of the substrate. The recovery of activity was observed when the mixture was treated at the pH range from 4.5 to 8.0.

Effect of Ferrous Ion on Fe^{++}-free and Fe^{++}-containing Enzymes—The effect of ferrous ion on the homogentisicase activity was reinvestigated using two

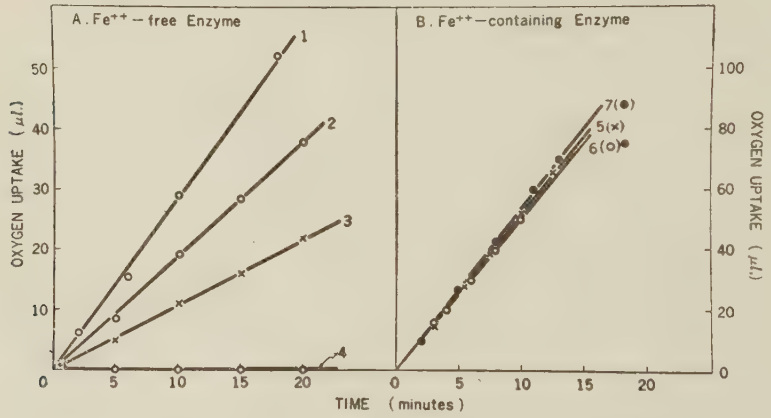


FIG. 1. Effect of ferreus ion on Fe^{++}-free and Fe^{++}-containing enzymes. Activity was measured manometrically and 30°, pH 6.5. Each vessel contained the enzyme preparation (A: Fe^{++}-free enzyme, 2.45 mg. protein; B: Fe^{++}-enzyme, 2.25 mg. protein, respectively), 2×10^{-3} *M* of homogentisic acid, 4×10^{-2} *M* of phosphate buffer, and distilled water in addition to the components specified below. Total volume was 2 ml. Gas phase was air. In systems 1, 2, 3, and 5, ferrous ion was preincubated with the enzyme for 10 minutes befor the reaction was initiated, and in system 6, ferrous ion and the substrate was added to the enzyme simultaneously.

1; $FeSO_4$, 1×10^{-3} *M*
2; $FeSO_4$, 5×10^{-4} *M*
3; $FeSO_4$, 2.5×10^{-4} *M*
4; None.
5; $FeSO_4$, 1×10^{-3} *M*
6; $FeSO_4$, 1×10^{-3} *M*
7; None.

kinds of preparations, *i.e.*, Fe^{++}-free and Fe^{++}-enzymes. The data obtained at pH 6.5 are shown in Fig. 1. The results show that the activity of Fe^{++}-free

enzyme was restored markedly by the addition of ferrous ion, but no activity was observed without its addition. Other metal ions such as Fe^{+++}, Cr^{+++}, Al^{+++}, Ni^{++}, Co^{++}, Zn^{++}, Pb^{++}, Sn^{++}, Mg^{++}, Ba^{++}, and MoO_4^{++}, at a concentra-

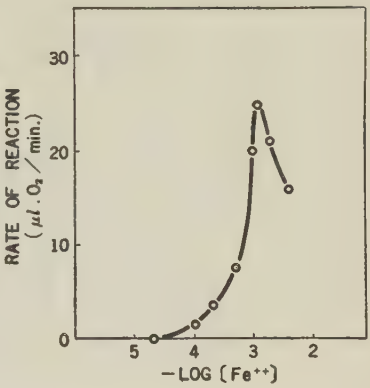


FIG. 2. Effect of ferrous ion concentration on homogentisicase activity.

Activity was measured manometrically at 30°, pH 6.6. Each vessel contained the Fe^{++}-free enzyme solution (3 mg. protein), 3.3×10^{-3} *M* substrate, 2×10^{-2} *M* tris buffer, $FeSO_4$ and distilled water to make final volume 2 ml.

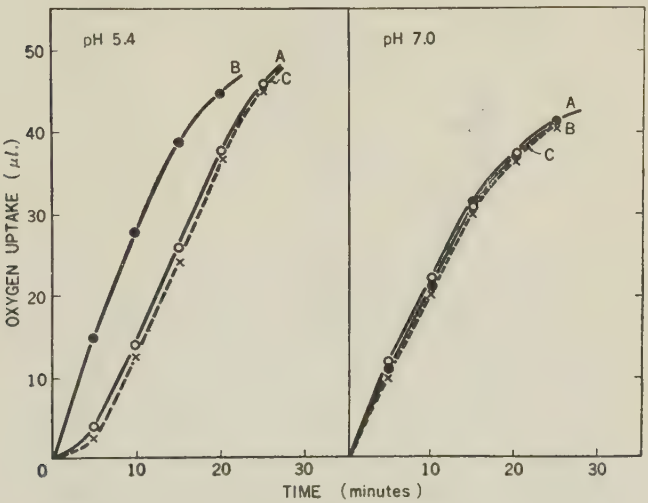


FIG. 3. Effect of preincubation of ferrous ion on Fe^{++}-free enzyme action.

Activity was measured manometrically at 30°. Each vessel contained the Fe^{++}-free enzyme solution (2.3 mg. protein), 5×10^{-4} *M* of $FeSO_4$, 3.3×10^{-3} *M* of homogentisic acid, 4×10^{-2} *M* of phosphate buffer, and distilled water to make a final volume of 2 ml. Gas phase air. In system A, ferrous ion and substrate, both of which were placed in two side arms separately, were added to enzyme solution simultaneously. In system B, enzyme solution was incubated with ferrous ion for 10 minutes before addition of substrate. In system C, enzyme solution was added to the mixture in which ferrous ion and substrate were preincubated together for 10 minutes.

tion of 10^{-3} *M*, could not replace the effect of ferrous ion*. These results confirmed the earlier observations (*1–3*). Fe^{++}-enzyme, however, had practically the same activity with and without the addition of ferrous ion at pH 6.5. It shows that Fe^{++}-enzyme retains the essential ferrous ion.

Fig. 2 shows the relationship, which was obtained at pH 6.6 using the Fe^{++}-free enzyme, between the activity and the concentration of ferrous ion. The optimum activity was observed at about 10^{-3} *M* of ferrous ion.

Feature of Activation by Ferrous Ion—In order to obtain detailed information concerning the activation of the enzyme by ferrous ion, the following experiments were carried out at various pH's. After the buffered Fe^{++}-free enzyme solution was incubated for 10 minutes both in the presence and absence of ferrous ion, the substrate alone or the substrate plus ferrous ion were added to the reaction medium and the initial rate was measured manometrically. The data obtained at pH 5.4 and 7.0 are shown in Fig. 3 as representatives. In the acid region (below pH 6.5), the initial rate was controlled by the order

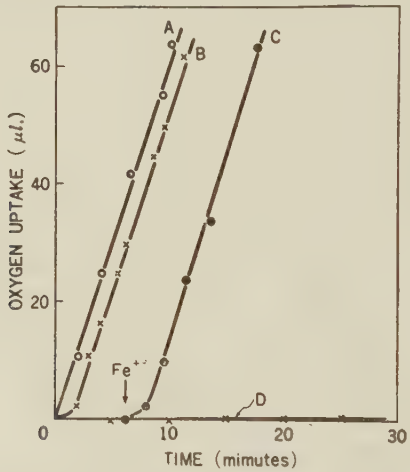


FIG. 4. Feature of ferrous ion of Fe^{++}-enzyme in acid region.

Activity was measured manometrically at 30°, pH 5.2. Each vessel contained the Fe^{++}-enzyme solution (2.25 mg. protein), 2×10^{-3} *M* of homogentisic acid, 1×10^{-3} *M* of ferrous ion, 1×10^{-3} *M* of glutathione, 4×10^{-2} *M* of phosphate buffer and distilled water to make final volume 2 ml. Gas phase was air. In system A, enzyme solution was mixed with ferrous ion for 10 minutes befor the substrate was added. In system B, substrate and ferrous ion were added simultaneously into the reaction mixture. In system C, ferrous ion was added 6 minutes after addition of substrate as indicated by the arrow. In system D, addition of ferrous ion was omitted. Substrate was added at zero time to enzyme solution in all cases.

* Both Mn^{++} and Cu^{++} were found to evoke apparent consumption of oxygen by Fe^{++}-free enzyme. It is, however, doubtful that this oxygen uptake was caused by the oxidation of homogentisate to maleylacetoacetate. This may well be due to the quinone formation from the substrate.

of addition of the two components, substrate and ferrous ion, to the system. If the two components were added simultaneously to the enzyme, an induction period was observed and full activity could be attained only after several minutes. On the other hand, when the enzyme was preincubated for 10 minutes in the presence of ferrous ion prior to the addition of the substrate, no induction period was observed even at acidic pH's. In the neutral and alkaline regions, no induction period could be observed even when the two components were added simultaneously.

Although the reaction of the Fe^{++}-enzyme took place at the same rate both in the presence and in the absence of added ferrous ion in neutral and alkaline regions, no reaction took place in the absence of added ferrous salts at higher acidity than pH 6.0. The data are shown in Fig. 4. The enzyme

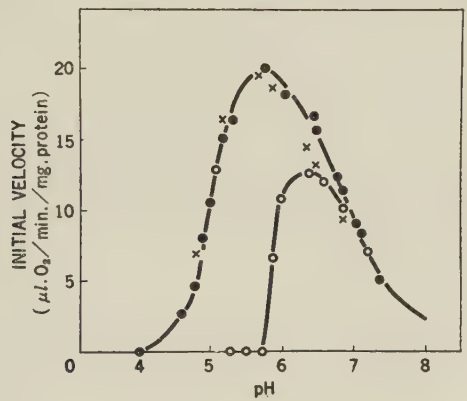


FIG. 5. Relationship between pH and activity

Enzymatic activity was measured manometrically at 30°. Each vessel contained the enzyme solution (Fe^{++}-enzyme; 2.25 mg, Fe^{++}-free enzyme; 1.4 mg. protein, respectively), 1×10^{-3} M of glutathione, 2×10^{-3} M homogentisic acid, 3.3×10^{-2} M of phosphate buffer and 1×10^{-3} M $FeSO_4$. Gas phase was air. Total volume was 2 ml.

——×——; Value was obtained using Fe^{++}-enzyme on the addition of ferrous ion.

——●——; Value was obtained using Fe^{++}-free enzyme in the presence of ferrous ion.

——○——; Value was obtained using Fe^{++}-enzyme without the addition of ferrous ion.

acquired its activity only when it was preincubated with 10^{-3} M ferrous salts. An induction period was also observed, as in the case of the Fe^{++}-free enzyme, when ferrous salts were added to the Fe^{++}-enzyme together with the substrate under such acidic conditions.

From this results, it is suggested that the combination reaction between enzyme protein and ferrous ion seems to be rapid in neutral and alkaline regions, but rather slow in acidic media, and that the essential ferrous ion of

the Fe^{++}-enzyme seems to dissociate from the enzyme protein in the acidic region.

In order to study the relationship among the enzyme protein, the substrate and ferrous ion, the initial velocity of the reaction in which the Fe^{++}-free enzyme was added to a preincubated mixture of ferrous ion and the substrate was followed in both acidic and neutral media. As may be seen in Fig. 3, it was found that the progress of the reaction was quite similar to that of the system in which the enzyme, ferrous ion and the substrate were simultaneously mixed. Thus, the possibility that a substrate-ferrous ion complex is the actual reactant in the homogentisicase reaction may be excluded.

pH-Activity Curve—The relationship between pH and activity was investigated manometrically using both the Fe^{++}-free and Fe^{++}- containing enzymes. The data obtained are shown in Fig. 5. The pH-activity curves of both samples were practically identical when they were preincubated with ferrous ion prior to the addition of the substrate. The optimum pH was found to be 5.8.

On the other hand, when the activity of the Fe^{++}-enzyme was measured at various pH values in the absence of added ferrous salts, a strikingly different pH-activity curve was obtained. The optimum activity appeared in the vicinity of pH 6.4, and the activity under this condition sharply fell at the acid side of pH 6.0. This difference can be expected from the data described in the previous section.

Other authors (*2*, *5*) have reported that the optimum pH of the enzyme isolated from rat liver is about 7.0. The discrepancies in the values, 5.8 and about 7.0, of optimum pH may have been due to the difference in the origin of the enzyme.

Effect of Iron-binding Agents—The effects of various iron-binding agents on the activity of the enzyme were studied. The assay was made manometrically by using Fe^{++}-enzyme in the presence and in the absence of an inhibitor.

As shown in Fig. 6, the activity of the enzyme was found to be strongly inhibited by *o*-phenanthroline and α,α'-dipyridyl both of which specifically bind with ferrous ion. The results obtained were in good agreement with the findings of other authors (*1–4*).

However, no inhibition was observed by the addition of thiocyanate which strongly reacts with ferric ion. Cyanide and azide, which are well known as inhibitors of hematin enzymes, also had little effect on the enzyme activity. It appears probable that the ferrous ion in the enzyme does not undergo any valency change in the course of the reaction.

Valency of the Iron Bound with the Enzyme—Using the Fe^{++}-enzyme prepared in the manner described already, it was attempted to examine by colorimetric analysis whether the essential iron contained in it undergoes any valency change during the reaction. For this purpose, the Fe^{++}-enzyme was allowed to react aerobically with the substrate in the absence of added ferrous salts. Control experiments were also run without the substrate. The level of ferrous ion in the reaction mixtures was determined at intervals by adding

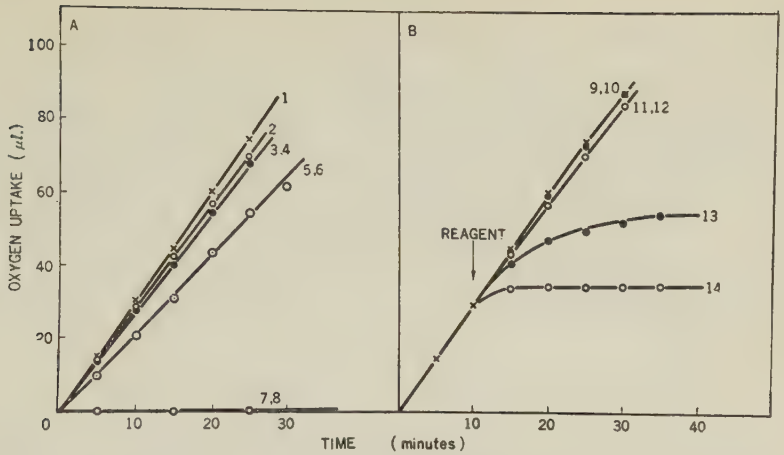


FIG. 6. Inhibition by iron-trapping agents.

Each vessel contained the Fe^{++}-enzyme solution (3 mg. protein), $2\times10^{-3}\,M$ of homogentisic acid, $3\times10^{-2}\,M$ of phosphate buffer, inhibitor indicated, and distilled water to make final volume 2 ml. All experiments were carried out manometrically at 30°, pH 6.5. Gas phase was air. In control system, inhibitor was omitted. A: Inhibitor was preincubated with enzyme at 30° for 30 minutes and then substrate was added. B; After the initiation of reaction, inhibitor was added to reaction mixture at the time indicated by arrow.

1: no inhibitor
2: KSCN, $4\times10^{-3}\,M$ or $2\times10^{-3}\,M$
3: KCN, $1.7\times10^{-3}\,M$
4: NaN_3, $1.7\times10^{-3}\,M$
5: KCN, $9\times10^{-3}\,M$
6: NaN_3, 9×10^{-3} M
7: *o*-phenanthroline, $1\times10^{-3}\,M$
8: α,α'-dipyridyl, $1.7\times10^{-3}\,M$
9: no inhibitor
10: KCNS, $4\times10^{-3}\,M$
11: KCN, $9\times10^{-3}\,M$
12: NaN_3, $9\times10^{-3}\,M$
13: α,α'-dipyridyl, $1.7\times10^{-3}\,M$
14: *o*-phenanthrolin, $1.7\times10^{-3}\,M$ or $1.0\times10^{-3}\,M$

TABLE II-A

Change in the Concentration of Ferrous Ion during Reaction

Each vessel contained the Fe^{++}-enzyme solution (11.8 mg. protein), $4\times10^{-2}\,M$ of tris buffer, pH 7.2, $3.3\times10^{-3}\,M$ of homogentisic acid, and distilled water to make the final volume 2 ml. In the control test, substrate was omitted. All reactions were carried out at 30° with shaking. *o*-Phenanthroline was added until the final concentration became 2 per cent. After 30 minutes the absorbance of each reaction mixture was measured at 500 mμ with a Coleman photometer. Final pH of these reaction mixtures was about 3.8. Extent of reaction which was measured by the oxygen comsumption occurring at each stage was expressed in percentage of the total oxygen consumption.

Reaction Time (*min.*)	Extent of reaction (%)	Absorbance at 500 mμ	
		Experimental (with substrate)	Control (without substrate)
0	9	—	0.085
6	27	0.100	0.110
20	75	0.100	0.094
35	100	0.095	0.089

an excess of *o*-phananthroline and measuring the color developed due to the formation of a ferro-phenanthroline complex. As shown in Table II-A, the concentration of ferrous ion in the preparation remained constant throughout the course of the reaction. This finding suggested that the iron remained unchanged in the ferrous state during the reaction; however, there was still a possibility that the apparent constancy of the ferrous iron concentration might have been effected by the balance between the oxidation of ferrous ion and the reduction of ferric ion. In order to ascertain this possibility, change in the concentrations of both ferric and ferrous ions, as might be affected by aeration and anaerobic incubation of the enzyme solution in the

TABLE II-B

Change in the Concentration of Ferrous and Ferric Ions

Each vessel contained the Fe^{++}-enzyme (6.5 mg. protein), 5×10^{-2} *M* of tris buffer, pH 7.2, 5×10^{-3} *M* of homogentisic acid in a final volume of 2.5 ml. In the control vessels, the substrate was omitted. The reactions were carried out at room temperature (~15°). Thunberg tubes were employed for anaeroble experiments, while aerobic experiments were conducted in open test tubes. In each experiment, the substrate was kept in contact with the buffered enzyme solution for 30 minutes and then (one of either) *o*-phenanthroline (2 per cent, ferrous iron) or NSCN (2 per cent, for ferric iron) was added, the reagents being added anaerobically in anaerobic experiments. The color developed was measured at 500 mμ in a Coleman photoelectric colorimeter after 30 minutes. The content of ferrous ion calculated from the experimental absorbances was about 1.5 μg.

Condition of Reaction		Absorbance at 500 mμ	
		Experimental (with substrate)	Control (without substrate)
Aerobic	Ferrous ion	0.060	0.055
	Ferric ion	0	0
Anaerobic	Ferrous ion	0.055	0.058
	Ferric ion	0	0

presence and in the absence of the substrate, were tested. As shown in Table II-B, it was, however, found that the level of ferrous ion did not suffer any change by these treatments, and only traces of ferric ion were detected under the experimental conditions employed. These observations seem to support the suggestion that no valency change in the essential ferrous ion takes place during the enzymatic reaction.

Exchange of Ferrous Ion during the Reaction—In order to obtain further information concerning the role of ferrous ion in the homogentisicase reaction, isotopic experiments were performed using Fe^{59} as a tracer. As can be seen from Table III, when the Fe^{++}-enzyme was brought into contact with Fe^{59}-

TABLE III

Exchange of Ferrous Ion during Reaction

Fe^{++}-enzyme solution (1 mg. protein) was incubated under aerobic and anaerobic conditions, with isotopic iron in $3\times10^{-2}\,M$ of tris buffer, pH 7.0, in the presence of homogentisic acid ($3.3\times10^{-3}\,M$) at 28° for 1 hour without shaking.

	Total counts			
	Before (*c.p.m.*)	After (*c.p.m./mg. protein*)		
		Aerob.	Anaerob.	Control (without substrate)
Free Fe^{59}	9060			
Fe^{59} bound to the enzyme	0	145	12	47

labeled iron salts in the presence of the substrate, an appreciable amount of exchange between the enzyme-bound ferrous ion and the exogenous Fe^{59} was observed under aerobic conditions. However, little, if any, exchange was detected when the reaction was carried out in the absence of molecular oxygen, a condition permitting no net reaction. The value found in the anaerobic experiment was approximately the same as that obtained in the control experiment in which no substrate was added.

Table IV shows the results of similar experiments conducted at different temperatures and for different intervals of time. It will be seen that the

TABLE IV

Effect of Temperature on the Exchange of Ferrous ion during Reaction

Fe^{++}-Enzyme solution (1 mg. protein) was incubated with isotopic iron in $3\times10^{-2}\,M$ of tris buffer, pH 7.0, at various temperatures in the presence of $3.3\times10^{-3}\,M$ of homogentisic acid for 1 hour without shaking. Reaction vessel was filled with air.

	Total counts			
	Before (*c.p.m.*)	After (*c.p.m./mg. protein*)		
Temperature (°C)		4°	28°	38°
Free Fe^{59}	9060			
Fe^{59} bound to the enzyme	0	81	145	205

Relationship between the reaction time and the degree of exchange of ferrous ion.

Fe^{++}-enzyme solution (1.5 mg. protein) was incubated with isotopic iron in $3\times10^{-2}M$ of tris buffer, pH 7.0, in presence of $3.3\times10^{-3}M$ of homogentisic acid at 30° with shaking. Reaction vessel was filled with air.

	Total Counts			
	Before (*c.p.m.*)	After (*c.p.m./1.5 mg. protein*)		
Time of Reaction (*min.*)		20 min.	90 min.	Control (90 min., without substrate)
Free Fe^{59}	1280			
Fe^{59} bound to the enzyme	0	136	454	79

amount of Fe^{59} bound to the enzyme protein increased in proportion to the increase in temperature and reaction time when the reaction was run under aerobic conditions. Furthermore, higher counts were always observed when the reaction mixture was shaken.

In these experiments the possibility that the isotopic iron was chelated by the substrate or by the reaction product and had escaped into the effluents without being trapped by the resin was excluded by the following experiments. When homogentisic acid ($3.3\times10^{-3}M$) or maleylacetyoacetic acid ($3.3\times10^{-3}M$) was brought into contact with the isotopic iron (total activity, 1280 c.p.m.) and then passed through the resin column, the radioactivity (40 and 48 c.p.m., respectively) detected in the effluents was negligibly small.

From these results it may be concluded that the exchange of ferrous ion occurs only when the enzymatic reaction is actually occurring, and that the rate of exchange increases with the velocity and/or the progress of the reaction.

DISCUSSION

Previously, Knox and Edwards (*6*) suggested, based on the results of their experiments, which showed the presence of inhibition by cyanide and azide on the enzymatic reaction, that homogentisicase might oxidize the substrate by changing the valency of the essential ferrous ion attached to the enzyme. However, the presence of inhibition, as obtained by them (*6*) and other authors (*2*, *3*), could not be confirmed in the present study. The following facts that ferric ion could not participate as an essential cofactor, inhibition was not observed by the addition of thiocyanate, and the amount of ferrous ion contained in the sample was essentially the same whether in the presence or in the absence of the substrate during the enzymatic reaction, seemed to suggest that no valency change in the iron occurred in the course of the enzymatic reaction. More conclusive information will be obtained by a magnetic experiment using a highly purified enzyme preparation.

From the results of the tracer experiments, it seems likely that the enzyme-bound ferrous ion becomes mobilized and exchanges with the exogenously

added Fe^{59} when the enzyme shows its function. A similar exchange of enzyme-bound metal ion with exogenously added labeled ion has also been reported with several copper- and iron-containing oxidases such as ascorbic acid oxidase (*11*), laccase* and pyrocatechase (*10*). Although the mechanism of exchange is still obscure, it seems that the binding force between the metal ion and the enzyme protein is decreased probably by the interaction which is induced when the substrate is oxidized on the surface of the enzyme.

SUMMARY

1. Two preparations of homogentisicase, namely Fe^{++}-free and Fe^{++}-containing enzymes, were partially purified from acetone-dried beef liver. The participation of ferrous iron in the homogentisicase reaction was confirmed.

2. In the presence of 10^{-3} *M* ferrous ion Fe^{++}-free and Fe^{++}containing enzymes showed identical pH-activity curves with an optimum at pH 5.8

3. In the acidic region the essential ferrous ion attached to the Fe^{++}-enzyme was removed from the enzyme protein. The ferrous ion added could rapidly combine with the enzyme protein in the neutral and alkaline regions, but it reacted slowly in the acidic region.

4. The exchange of the bound ferrous ion with exogenous Fe^{59} occurred only when the enzyme showed its function.

The author wishes to express her deep gratitude to Prof. M. Suda for his kind guidance throughout this work and to Prof. H. Tamiya, Dr. Y. Ogura and Dr. R. Sato for their valuable advice and suggestions.

REFERENCES

(*1*) Suda, M., and Takeda Y., *J. Biochem.*, **37**, 381 (1950)
(*2*) Crandall, D. I., *Federation Proc.*, **12**, 192 (1953)
(*3*) Schepartz, B., *J. Biol. Chem.*, **205**, 185 (1953)
(*4*) Knox, W. E., quoted in D. I. Crandall, A symposium on amino acid metabolism, Baltimore, p. 836 (1955)
(*5*) Crandall, D. I., *J. Biol. Chem.*, **212**, 565 (1955); A symposium on amino acid metabolism, Baltimore, p. 867 (1955)
(*6*) Knox, W. E., and Edwards, S. W., *J. Biol. Chem.*, **216**, 479 (1955); A symposium on amino acid metabolism, Baltimore p. 836 (1955)
(*7*) Suda, M., *Med. J. Osaka Univ.*, **8**, Supplement, p. 57 (1958)
(*8*) Sutherland, W. E., Cori, C. F., Haynes, R., and Olsen, N. S., *J. Biol. Chem.*, **180**, 825 (1949)
(*9*) Barken, G., and Walker, B. S., *J. Biol. Chem.*, **135**, 37 (1940)
(*10*) Tokuyama, K., Suda, M., and Shimomura, Y., *Proc. Intern. Symposium on Enzyme Chem., Tokyo and Kyoto, 1957*, Maruzen (Tokyo), p. 197 (1958)
(*11*) Joselow, M., and Dawson, C. R., *J. Biol. Chem.*, **191**, 1 (1951)
(*12*) Nagao, K., and Kiyokawa, M., *Osaka Igaku-kai Zasshi*, **34**, 661 (1935)
(*13*) Tsuchihashi, K., *Biochem. Z.*, **140**, 63 (1923)
(*14*) Knox, W. E., and Edwards, S. W., *J. Biol. Chem.*, **216**, 489 (1955)

* T. Ohmura; personal communication.

The Journal of Biochemistry, Vol. 46, No. 10, 1959

PREPARATION OF L-KYNURENINE FROM RAT FUR

By AKIRA HASHIMOTO

(From the Department of Biochemistry, Faculty of Medicine, Nagoya University, Nagoya)

(Received for publication, March 16, 1959)

Hashimoto and others have studied previously on the fluorescent substances contained in mammalian skin and fur, and one of them was considered to be L-kynurenine (*1*, *2*). Since it was expected that a large amount of this substance can be easily prepared from rat fur, the following extraction methods were compared at first; the material was i) directly, ii) after grinding with water or iii) after washing with acetone-ether, extracted with water at 80° for 30 minutes. The extracts were deproteinized by trichloracetic acid and kynurenine contents were determined by the method of Berg (*3*). Those extraction methods gave similar results. However, for the preparation of kynurenine the previous washing out of tallow by acetone-ether was recommended. The grinding of fur was then not necessary. The quantitative extractibility of kynurenine by hot water was proved as follows. The amount of anthranilic acid determined as the decomposition product of kynurenine after direct heating of the raw material with 2 *N* NaOH was similar to that in the water extract, obtained from the same amount of material and alkali-treated afterwards.

About 10 g. of fur washed by acetone-ether mixture was stirred with 1 liter of water at 80–90° for 60 minutes. The yellowish extract obtained by filtration was acidified to pH 3.5 by adding few ml. of 1 *N* H_2SO_4 and the precipitates formed were discarded by filtration. The filtrate was shaken with 200 ml. of ether and the aqueous layer, which was now slightly yellowish and almost clear, was concentrated, usually to 200–300 ml., under reduced pressure in a current of nitrogen, saturated by ammonium sulfate and twice extracted with 1/10 volume of liquid phenol. To the combined phenol layer about 50 ml. of 2 *N* H_2SO_4 and then a large amount of ether were added. The brown aqueous layer was washed with ether, and poured into the same volume of 10 per cent mercuric sulfate dissolved in 2 *N* H_2SO_4. The precipitates were centrifuged after standing 48 hours in the cold, washed three times with 2 *N* H_2SO_4, suspended in about 50 ml. of water and treated with hydrogen sulfide. The clear filtrate was concentrated under reduced pressure in a current of nitrogen until crystals began to appear and four volumes of hot ethanol were added. Almost colourless, needleshaped crystals obtained after standing 24

hours in the cold were recrystallized by decoloring with charcoal from 80 per cent ethanol. The yield was between 40 to 50 mg. from 10 g. of material. The substance became coloured again, however, when it remained several hours in aqueous or alcoholic solution.

For the identification, the following properties of the product were compared with a synthetic L-kynurenine sulfate (*4*);

1. The ultraviolet absorption was measured by a Beckman spectrophotometer model DU, using 0.1 *M* phosphate buffer, pH 7.0, as the solvent. The absorption maxima were found at 230, 257, and 360 mμ. The ratio of the optical densities at these wave length was 1.0/0.39/0.24.

2. It melted at about 170° and decomposed at about 190°.

3. Ascending paper chromatography performed using several kinds of developing solvent gave a single fluorescent or ninhydrin positive spot of the R_f value identical with the synthetic sample.

4. Nitrogen content, determined by the method of Lubochinsky (*5*), was 8.6 per cent.

5. The pH-fluorescence curve of the solution prepared by neutralization by $Ba(OH)_2$ showed two peaks at about pH 4 and 12.

6. The fluorescence spectrum of the sample solution neutralized with 0.01 *N* NaOH was analyzed. The maximum was found at 490 mμ.

These results were all identical with synthetic sample. The product was identified as L-kynurenine sulfate.

REFERENCES

(*1*) Hashimoto, A., *Vitamins*, **13**, 419, (1957)
(*2*) Ishiguro, I., Hashimoto, A., and Tsuboi, S., *J. Japan. Biochem. Soc.*, **31**, 218 (1959)
(*3*) Mason, M., and Berg, C. P., *J. Biol. Chem.*, **195**, 515, (1952)
(*4*) Warnell, J. L., and Berg, C. P., *J. Am. Chem. Soc.*, **76**, 1708 (1954)
(*5*) Lubochinsky, B., and Zalta, J., *Bull. soc. chim. biol.*, **36**, 1363 (1954)

The Journal of Biochemistry, Vol. 46, No. 10, 1959

CHROMATOGRAPHIC STUDIES ON HEAT DENATURATION OF BOVINE PLASMA ALBUMIN SUBFRACTIONS*

The authors studied the rates of denaturation and renaturation reactions of Armour's bovine plasma albumin at pH 6.8 over the temperature range 54~60° employing change of chromatogram as the criterion of heat denaturation

0.2 per cent bovine plasma albumin solution (0.02 *M* sodium phosphate buffer of pH 6.8) was heated at 54.1°, 57.1° and 60.0°. After heating of various times, protein solution was immersed quickly into ice-water and was chromatographed. The heated protein solution was not cloudy. Chromatographic analysis was carried out by using hydroxylapatite column developed by Tiselius (*1*). However, the authors used more coarse hydroxylapatite which was prepared by authors' method (*2*). Three subfractions were eluted by 0.07 *M*, 0.11 *M* and 0.40 *M* of sodium phosphate buffer pH 6.8, respectively (*1*).

Fig. 1 showed the changes of three subfractions of heat denatured albumin (57.1°, 60.0°) against time. The change of subfraction eluted by 0.07 *M* buffer (1-st subfraction) was more quickly approached an equilibrium than those of other subfractions. The percentage of the subfraction eluted by 0.11 *M* buffer (2-nd subfraction) was constant (20 per cent) at 54.1°, 57.1° and 60.0 over the time range 100~300 minutes. In the case of the lower part of Fig. 1, the 2-nd subfraction was gradually decreased after about 80 minutes. The authors used I^{131}-bovine plasma albumin for the study of denaturation at 60°. Specific activities of 1-st, 2-nd and 3-rd subfractions were 1.90×10^3, 12.5×10^3, 3.2×10^3 c.p.m. per mg. of protein, respectively**. As shown in Fig. 2, percentage and specific activity of 2-nd subfraction were almost constant for 60 minutes at 60.0°. The difference in heat stability of two subfractions against heat was similar to that of hemoglobin in blood which was indicated by a break in the denaturation velocity (*3*).

Chromatographic change of the 1-st subfraction took place according to equation (I) (*4*).

$$l_n\ (a-a_e) = -(k_1+k_2)\,t + \text{constant} \qquad \text{(I)}$$

$$K=\frac{k_1}{k_2}=\frac{a_0-a_e}{a_e} \qquad \text{(II)}$$

(a_0, a and a_e: Concentrations of the 1-st subfraction at time 0, t and at an equilibrium, respectively. k_1 and k_2: rate contants of denaturation and re-

* This investigation was supported in part by a grant from the Ministry of Education in 1958.

** This method will be published in this journal.

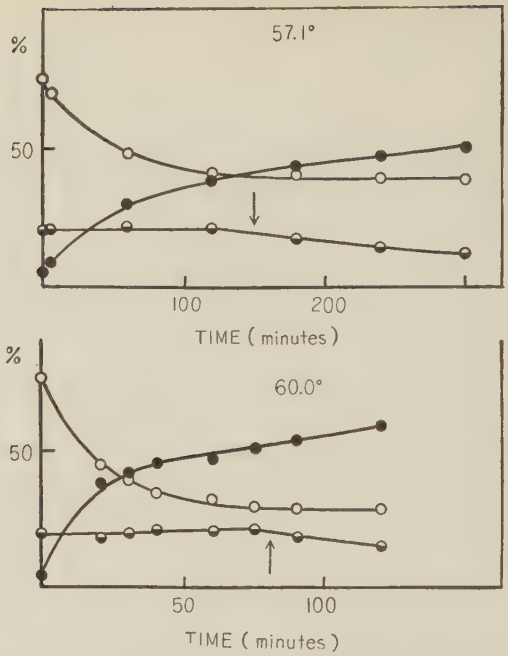


FIG. 1. The plot of percetages of three subfraction which were eluted by 0.07 *M* (○), 0.11 *M* (◒) and 0.40 *M* (●) of sodium phosphate buffer pH 6.8 against time (minute). The heat denaturation was carried out at 57.1±0.05° (the upper diagram) and 60.0±0.05° (the lower one).

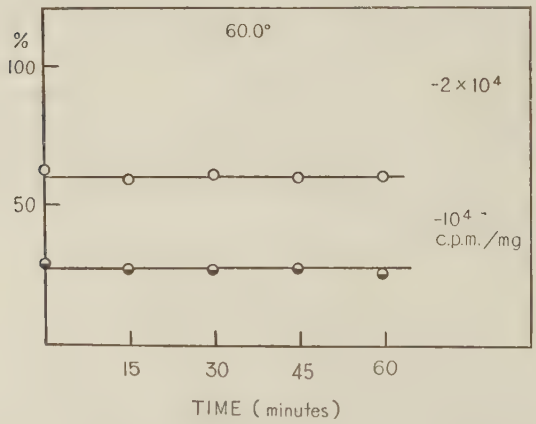


FIG. 2. The plot of percentage (◒) and specific activity (○) of 2-nd subfraction against time at 60.0°. The left and right ordinates show percentage and count per minute per mg. of protein, respectively.

natration reaction, respectively). Equilibrium consts at 60.0°, 57.1° and 54.1° were 1.621, 0.949 and 0.310, respectively. Enthalpy (ΔH) and entropy (ΔS) of reaction were 64 k cal/mol and 193 E.U., respectively. Gibbs' free energy (ΔG) was zero at 49°. These values were similar to those of denaturation of soybean trypsin inhibitor (*5*) and chymotrypsimogen (*6*). Activation enthalpy (ΔH_1^*) and entropy (ΔS_1^*) of denaturation reaction were 120 k cal/mole and 232 E.U., respectively. ΔH_2^* and ΔS_2^* of renaturation reaction were 39 k cal/mole and 39 E.U., respectively.

The authors wish to thank Prof. Akira Inouye (Department of Physiology, Faculty of Medicine, Kyoto University) and Prof. Goro Kawabata (Department of Physiology, Yamaguchi Medical College) for their excellent discussions. The authors also express their gratitude to Institute for Tuberculosis of Kyoto University and Department of Biochemistry, Yamaguchi Medical College for their permission to use Beckman spectrophotometer.

REFERENCE

(*1*) Tiselius, A., Hjert'n, S., and Levin, Ö., *Arch. Biochem. Biophys.*, **65**, 132 (1956)
(*2*) Sogami, M., and Takeo, Y., *J. Biochem.*, In press
(*3*) Haurowitz, F., Hardin, H.I., and Dicks, M., *J. Phys. Chem.*, **58**. 103 (1954)
Haurowitz, F., *Z. physiol. Chem.*, **186**. 141 (1930)
(*4*) Johnson, F. H., Eyring, H., and Polissar M, J., The Kinetic Basis of Molecular Biology, John Wiley and Sons, Inc., New York. p. 245 (1954)
(*5*) Kunitz, M., *J. Gen. Physiol.*, **32**, 241 (1948)
(*6*) Eisenberg, M. A., and Schwert, G. W., *J. Gen Physiol.*, **34**, 583 (1951)

Department of Physiology
Faculty of Medicine
Kyoto University, Kyoto
MASARU SOGAMI*

Department of Physiology
Yamaguchi Medical College
Ube
SHIGEJI TAKEMOTO

* Present Address: *Department of Physiology Yamaguchi Medical College, Ube.*

(Received for publication, August 17, 1959)

昭和 32 年 7 月 9 日 第三種郵便物認可
第 46 巻 第 10 号 昭和 34 年 10 月 25 日 発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 10, October 25, 1959
Vol. 46 No. 10

CONTENTS